THE EXPRESSION OF Fos, Jun AND AP-1 DNA BINDING ACTIVITY IN RAT SUPRAOPTIC NUCLEUS NEURONS FOLLOWING ACUTE VERSUS REPEATED OSMOTIC STIMULATION

1995

WANG

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

GRADUATE EDUCATION
(301) 295-3913

APPROVAL SHEET

Title of Dissertation: "The Expression of Fos, Jun and AP-1 DNA Binding Activity in Rat Supraoptic Nucleus Neurons Following Acute Versus Repeated Osmotic Stimulation"

Name of Candidate: Ke Wang
Doctor of Philosophy Degree
22 June 1995

Dissertation and Abstract Approved:

Committee Chairperson	6-22-95 Date
Committee Member	6-22-95 Date
Committee Member	6 21-95 Date
Committee Member	6-20-95 Date

The author hereby certifies that the use of any copyrighted material in the dissertation manuscript entitled:

"The Expression of Fos, Jun and AP-1 DNA Binding Activity in Supraoptic Nucleus Neurons Following Acute *Versus* Repeated Osmotic Stimulation"

beyond brief excerpts is with the permission of the copyright owner, and will save and hold harmless The Uniformed Services University of the Health Sciences from any damage which may arise from such copyright violations.

Ke Wang
Department of Anatomy and Cell Biology
Uniformed Services University
of the Health Sciences

ABSTRACT

Title of Dissertation: The Expression of Fos, Jun and AP-1 DNA Binding Activity in Supraoptic Nucleus Neurons Following Acute *Versus* Repeated Osmotic Stimulation

Ke Wang, Doctor of Philosophy, 1995

Dissertation directed by: Joseph T. McCabe, Ph.D.
Associate Professor
Anatomy & Cell Biology Department

Fos and Jun are protein products of their respective proto-oncogenes, c-*fos* and c-*jun*. Nuclear concentrations of both proteins rapidly, but transiently, rise after the presentation of a variety of stimuli under *in vitro* and *in vivo* conditions. Fos and Jun can form heterodimeric proteins that act as transcription factors to regulate late response genes.

Previous work has demonstrated that hypertonic saline injection results in Fos expression in supraoptic nucleus (SON) neurons that mediate body water homeostasis. Since Fos is thought to act mostly in conjunction with Jun, I first developed a double-label fluorescence immunocytochemical procedure that permits simultaneous visualization of neurons expressing Fos and Jun. After hypertonic saline administration, the immunostaining of c-Fos and c-Jun was coexistent in the majority of magnocellular neurons of SON.

This coordinated c-Fos and c-Jun immunostaining occurred within 30 min, peaked at 90 to 120 min, and disappeared by 4 hr.

Northern blotting and *in situ* hybridization was used to study changes in c-*fos* and c-*jun* mRNA levels after acute hypertonic saline injection. c-*fos* and c-*jun* mRNA expression occurred within 5 min, peaked at 30 to 60 min, and disappeared by 3 hr. The latency for increased levels of c-*fos* mRNA was faster than for c-*jun* mRNA, and the response was stronger and lasted for a longer period of time.

Compared with results after an acute hypertonic saline injection, both protein (c-Fos, c-Jun) and mRNA expression (c-*fos*, c-*jun*) were dramatically decreased when rats received repeated hypertonic saline injection. The decrease for of c-*jun* was greater than that of c-*fos*.

Gel shift DNA-protein binding assay indicated that SON extracts could bind to AP-1 oligomer. The incubation of tissue extracts obtained from osmotically stimulated rats with c-Fos antisera resulted in a supershifted binding pattern.

The Expression of Fos, Jun and AP-1 DNA Binding Activity in Rat Supraoptic Nucleus Neurons Following Acute *Versus* Repeated Osmotic Stimulation

by

Ke Wang

Dissertation Submitted to the Faculty of the Department of Anatomy and Cell Biology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 1995

DEDICATION

To my mother, Jin-hua Wang, and my father, Yu-xin Wang, who have given me a lifetime of love and incredible support, who wanted me to achieve this goal as much as I did. Thank you, Mom and Dad, for the example you have set by being there for me in so many ways.

To my beloved husband Kan Liu, who has been my strength and driving force, who gave me confidence, and helped me through many dark tunnels. Without his constant love, companionship, and encouragement, the completion of this thesis would not have been possible. This dissertation is as much a credit to his efforts as it is to mine.

To my son, Clive, and my daughter, Diana, for their consideration, understanding and sacrifice while I have pursued my dreams.

To my sister, Jin Wang, for her love and support.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to:

Dr. Joseph T. McCabe - for being my advisor and playing an integral part in my development as a scientist. He has given freely and always of his time, talent and support. Thank you for your time, the effort and the patience. I will miss your expert guidance, insightful suggestions and kind personality.

Dr. Juanita J. Anders - for being a great committee chairperson. Thank you for your encouragement and always being available and listening to me when times were less than optimal.

Dr. David C. Beebe - for being my committee and helpful suggestions , for the generous use of his Lab instruments. Thank you for your time.

Dr. Rosemary C. Borke - for her support, generosity which have been invaluable to me. Thank you for your time and energy.

Dr. Griemley - for agreeing to sit on my committee and providing the advice.

Drs. Steven Bassnett, Rita Dhawan, Chun-ling Gao, Ronald Labiche, and Jay Potts - for their helpful suggestions and molecular technique assistance.

Drs. Michael Sheridan, Mark R. Adelman, Regina C. Armstrong, Jill Carrington, James N. Coulombe, Sharon Juliano, ASAF Keller, Kathryn J. Lynch, Donald B. Newman, Linda Porter, Mark D. Rollag - for their advice and help for my graduate study.

Mary L. Thomson and Dawn Walker - for their secretarial assistance.

Ling Young, Ling Ou-yong and Hui Fong - for their friendships, and support. Thank you for helping me through the toughest of times.

Dr. Xian Li - for being good friend in need, for having a sympathetic ear, and for sharing many enlightening conversations.

Dr. Wu Ma - last, but above all not least, for his friendship, extensive help, and encouragement. Thank you for your time, energy and patience.

TABLE OF CONTENTS

List of Tables

Table Page

List of Figures

Chapter 1.

Introduction

Proto-Oncogenes

Proto-oncogenes are presently the subject of intense research activity (Stehelin et al., 1976; Bishop & Varmus, 1982; Bishop, 1983 and 1987; van Beveren & Verma, 1985; Angel & Karin, 1991; Piechaczyk & Blanchard, 1994). Major research efforts have focused on elucidating the physiological roles of proto-oncogenes in transformed and normal cells, and it appears their function can be classified into three categories: growth factors and receptors, mediators of intracellular signal transduction pathways, and regulators of gene expression (Bishop, 1987). It is likely that interaction and cooperation among the products of the different classes of proto-oncogenes plays a cardinal role in cell growth, differentiation, and development. Since the products of a number of proto-oncogenes have been found to reside in the nucleus, these proto-oncogenes are thought to play a role in gene expression. Oncogenes that encode nuclear proteins include *myc*, *myb*, *fos*, *jun*, *erb A*, *ski*, *rel*, *E1A*, *SA40* large T antigen, and polyoma large T antigen. Products of nuclear

oncogenes like *jun* and *erbA* have been identified as transcription factors; the former, for example, being a constituent of the phorbol-ester-responsive factor AP-1, while the latter is the thyroid hormone receptor (Sap et al., 1986; Weinberger et al., 1986; Bohmann et al., 1987; Angel et al., 1988). Other nuclear oncogenes like *fos*, *myc* and *rel* may act as transregulators of transcription by interaction with other nuclear factors (Setoyama et al., 1986; Distel et al., 1987; Verma & Sassone-Corsi, 1987; Rauscher et al., 1988a).

The Oncogenes c-*fos* and c-*jun*

The oncogenes *fos* and *jun* were first described as the transforming genes carried by the FBJ murine sarcoma virus (Curran and Teich, 1982) and avian sarcoma virus 17 (Maki et al., 1987), respectively. Their normal cellular homologs, c-*fos* and c-*jun*, are expressed at relatively low levels in the majority of cell types. However, they are induced transiently to very high levels by a great variety of extracellular stimuli (for reviews, see Curran, 1988; Cohen and Curran, 1989). Both c-*fos* and c-*jun* are members of the set of genes known as cellular immediate-early genes (Curran and Morgan, 1987; Lau and Nathans, 1987). This gene set has been defined operationally by the fact that expression is

induced rapidly by a range of extracellular stimuli, even in the presence of protein synthesis inhibitors (Lafarga et al., 1993). Thus, in unstimulated cells, the protein factors needed for initiating transcription are present in the cell and become activated by post-translational modification(s), such as phosphorylation, reduction/oxidation reaction and glycosylation (Morgan & Curran, 1991; Hunter et al., 1992; Karin, 1994).

Cellular immediate-early genes have been proposed to function in coupling short-term stimuli to long-term changes in cellular phenotype (reviewed in Sheng et al., 1990; Morgan and Curran, 1991). Genes whose transcription is activated rapidly and transiently within minutes of stimulation (the cellular immediate early genes), includes *c-fos* and *c-jun* (Greenberg et al., 1985; Morgan and Curran, 1986; Bartel et al., 1989; Barzilai et al., 1989), and it is proposed they orchestrate the expression (and repression) of other sets of genes. The latter are referred to as late response genes and are thought to be dependent upon new protein synthesis (Merlie et al., 1984; Castellucci et al.,1988; Goldman et al.,1988; Barzilai et al., 1989; Offord and Catterall, 1989; Klarsfeld et al.,1989). It has been proposed that the immediate early genes encode regulatory proteins that control the expression of late response genes. The products of the late response genes then serve more

specific effector functions in cell signalling, adaptation, metabolism, growth, and differentiation (Morgan and Greenberg, 1990).

c-Fos and c-Jun as Regulators of Gene Transcription

The protein products of c-*fos* and c-*jun* are c-Fos and c-Jun, respectively. These two proteins interact as a heterodimeric protein complex and bind specific regulatory DNA sequence motifs; the most documented binding motifs being, AP-1 (**a**ctivator **p**rotein-1) binding to the consensus sequence, TGACTCA, and another regulatory site, CRE (**c**yclic AMP **r**esponsive **e**lement), binding to the sequence, TGACGTCA (for review, see Curran and Franza, 1988).

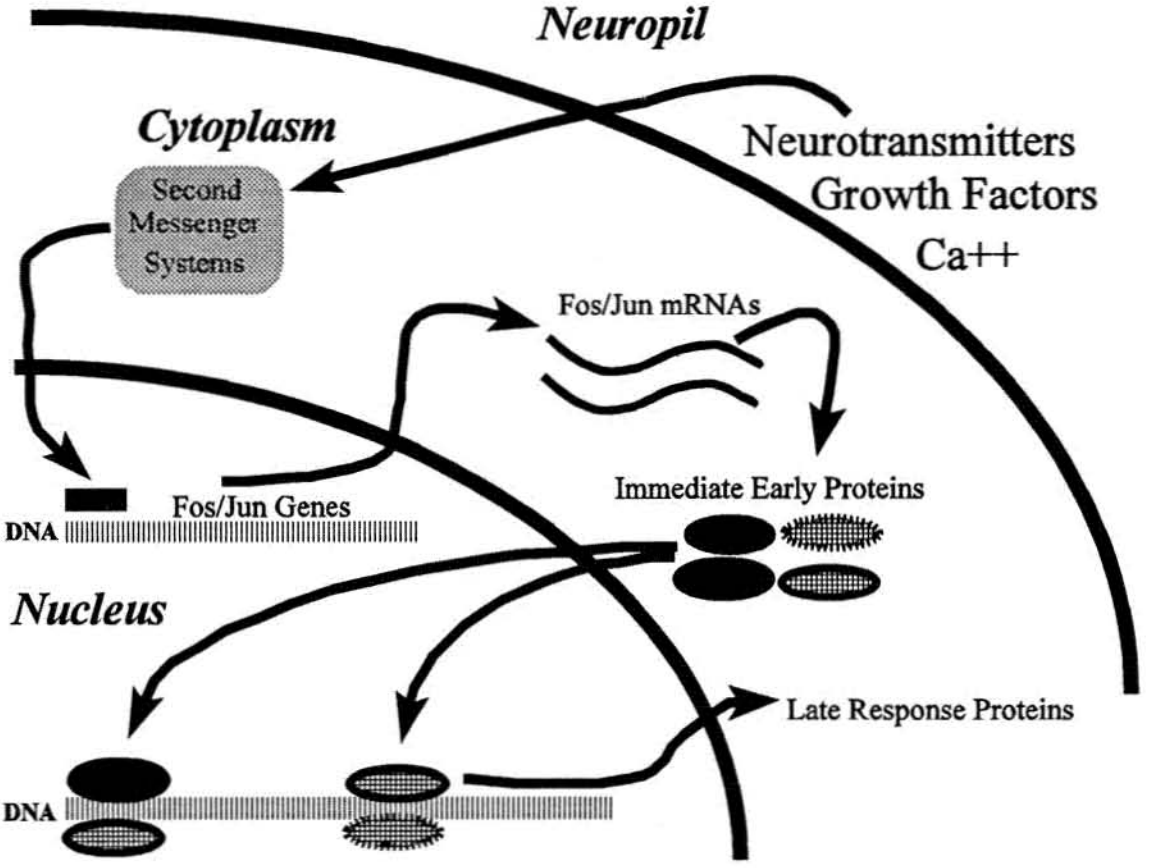
Both *fos* and *jun* are members of gene families. The other family members are also induced by extracellular stimuli, and they are all capable of forming heterodimeric and homodimeric complexes with similar, although not identical, DNA-binding specificities (Cohen and Curran, 1988; Ryder et al., 1988 and 1989; Cohen et al., 1989; Zerial et al., 1989). The *fos* family is known to presently include c-*fos*, *fos*B, and *fra*-1 and -2. *fra* refers to **F**OS-**r**elated **a**ntigen. Western blot experiments and employment of less stringent nucleic acid hybridization procedures allowed

investigators to discover these products closely related to FOS (Cohen et al., 1989; Franza et al., 1987; Zerial et al., 1989). All of the Jun family members, c-*jun*, *jun-B* and *jun-D*, can form homodimeric and heterodimeric complexes with each other, whereas the Fos family members, c-*fos*, *fra-1* and *fosB*, only form heterodimeric complexes with Jun-related proteins (Nakabeppu et al., 1988; Rauscher et al., 1988b).

The AP-1 site of many genes is responsive to TPA (12-*O*-tetradecanoylphorbol 13-acetate: Angel et al., 1987; Lee et al., 1987a, b, c, and 1989), and therefore is often referred to as TRE (**T**PA-**r**esponsive **e**lement). The TRE DNA sequence motif is an upstream transcriptional control element for many genes, and it contributes both negatively and positively to basal and regulated rates of transcription. It is generally known that proteins encoded by the *fos* and *jun* gene families function as "intermediary transcription factors", because they are responsive to a variety of second-messenger signals including the breakdown of phospholipids, activation of protein kinase-C, and intracellular release of calcium ions. Hence, Curran and his colleagues viewed them as "third messengers" (1990) in a stimulus-transcription coupling cascade (Figure. 1).

Figure. 1 A simplified representation of the steps that follow stimulation of neurons by neurotransmitters, calcium, and growth factors (adapted from Curran et al., 1990). Second messengers, perhaps all eventually mediating a protein kinase-C response, activate messengers that promote the transcription of Fos and Jun gene-family members. *fos* and *jun* immediate-early mRNAs are translated in the cytoplasm, and Fos and Jun proteins migrate to the nucleus. In the nucleus, Fos and Jun proteins form heterodimers, which then bind to AP-1 sites located in the upstream transcriptional control regions of many genes. Immediate early proteins then cause increased (and in some cases, decreased) transcription of late response genes. Several recent reviews outline the complex signal pathways that control the transcription of *fos* and *jun* (Ghosh et al., 1994; Hunter and Karin, 1992; Karin, 1994: Piechaczyk and Blanchard, 1994).

Schematic Diagram of the Cascade of Events from Cell Stimulation to the Regulation of Late Response Genes by Fos and Jun



c-*fos* and c-*jun* in the Nervous System

Nerve cells, with their unique morphological and excitable properties, have specialized functions for the reception, transmission, and storage of information. It has long been recognized that trans-synaptic signals cause rapid responses in neurons from milliseconds (e.g., opening of ligand-gated channels) to seconds and minutes (e.g., second messenger-mediated events). Recent studies have revealed that trans-synaptic activation also elicits slower, long-term responses in neural cells that are correlated with the induction of new programs of gene expression (reviewed in Comb et al., 1986; Goelet et al., 1986; Black et al., 1987; Morgan and Curran, 1988).

Numerous recent studies have demonstrated that the immediate early genes are induced during stimulation of the nervous system, thus supporting the idea that these genes take part in nerve cell responses. In the central nervous system, c-*fos* or c-*jun* expression appears in response to diverse stimuli such as seizures (Dragunow et al., 1987; Morgan et al., 1987; Sagar et al., 1988b; Sonnenberg et al., 1989; Gass et al., 1992), sensory stimulation (Hunt et al., 1987), opiate withdrawal (Hayward et al., 1990), gonadal steroid administration (Hoffman et al., 1990; Insel et al., 1990), and electrical stimulation (Herdegen et al., 1991).

In vertebrates, the activation of gene expression in neurons has been shown to be critical for the development of a learning-related long-term facilitation of neuron firing patterns (Montarolo et al.,1986).

The Hypothalamo-Neurohypophysial System

The neurohypophysial system (or magnocellular neurosecretory system) is composed of approximately 15,500 magnocellular neurons. These neurons are localized in several hypothalamic brain areas but are concentrated in two hypothalamic nuclei- the supraoptic nucleus (SON; the two SON contain about 4500 magnocellular neurons), and the paraventricular nucleus (PVN; about 11,000 neurons). Additional cells are distributed in the anterior commissural nucleus (ACN), and as a collection of "satellite" or "accessory" neurons distributed throughout the midlateral hypothalamus (Swaab et al., 1975; Rhodes et al., 1981; Silverman et al., 1983; Ju et al., 1986;). In spite of their different locations in the hypothalamus, most of these neurons project their terminals to the neurohypophysis; the posterior lobe of the pituitary (Ju et al., 1986; Swanson, 1986). Anatomical studies of this group of hypothalamic neurons has provided a detailed description of neuron location as well as afferent and efferent connectivity

(Morris et al., 1978; Rhodes et al., 1981; Silverman et al., 1983; Weindl 1985; Ju et al., 1986; Palkovits 1986; Swanson 1986). Besides synthesis of the neurohypophysial hormones, vasopressin (VP, antidiuretic hormone) and oxytocin (OT), these neurons produce other neuropeptides and neurotransmitter substances (Swanson et al., 1983; Brownstein et al., 1986; Meister, 1993). For example, vasopressinergic magnocellular neurons synthesize the opioid peptides, leu-enkephalin and dynorphin, as well as galanin, and dopamine; oxytocinergic magnocellular neurons make three other peptides: cholecystokinin, met-enkephalin, and corticotropin-releasing factor. With respect to VP and OT, endocrinological and molecular biological investigations have analyzed how these hormones are synthesized, processed, packaged, transported, and released from the pituitary (Morris et al., 1978; Brownstein 1983; Schmale et al., 1983; Castel et al., 1984; Gainer et al., 1985; Ivell and Richter, 1984; Altstein et al., 1988), and have described how bodily state, hormone condition, and electrical and chemical stimulation affects synthesis and release (Greep and Astwood, 1974; Morris et al., 1978; Retting et al., 1982; Brownstein, 1983; Schmale et al., 1983; Castel et al., 1984; Doris, 1984; Ivell and Richter, 1984; Reichlin, 1984; Reid and Schwartz, 1984; Amico and Robinson, 1985; Gainer. et al., 1985; Ganten and Pfaff, 1985 and 1986; Schrier, 1985). Furthermore, functional investigations have demonstrated how

these hormones then subserve important homeostatic functions, including water balance, blood pressure control, stress, parturition and lactation (Robertson, 1977; Amico and Robinson, 1985; Larson, 1985; Schrier, 1985). However, little is known concerning the actual intracellular mechanisms that regulate these responses.

The Regulation of Body Fluid Osmolality

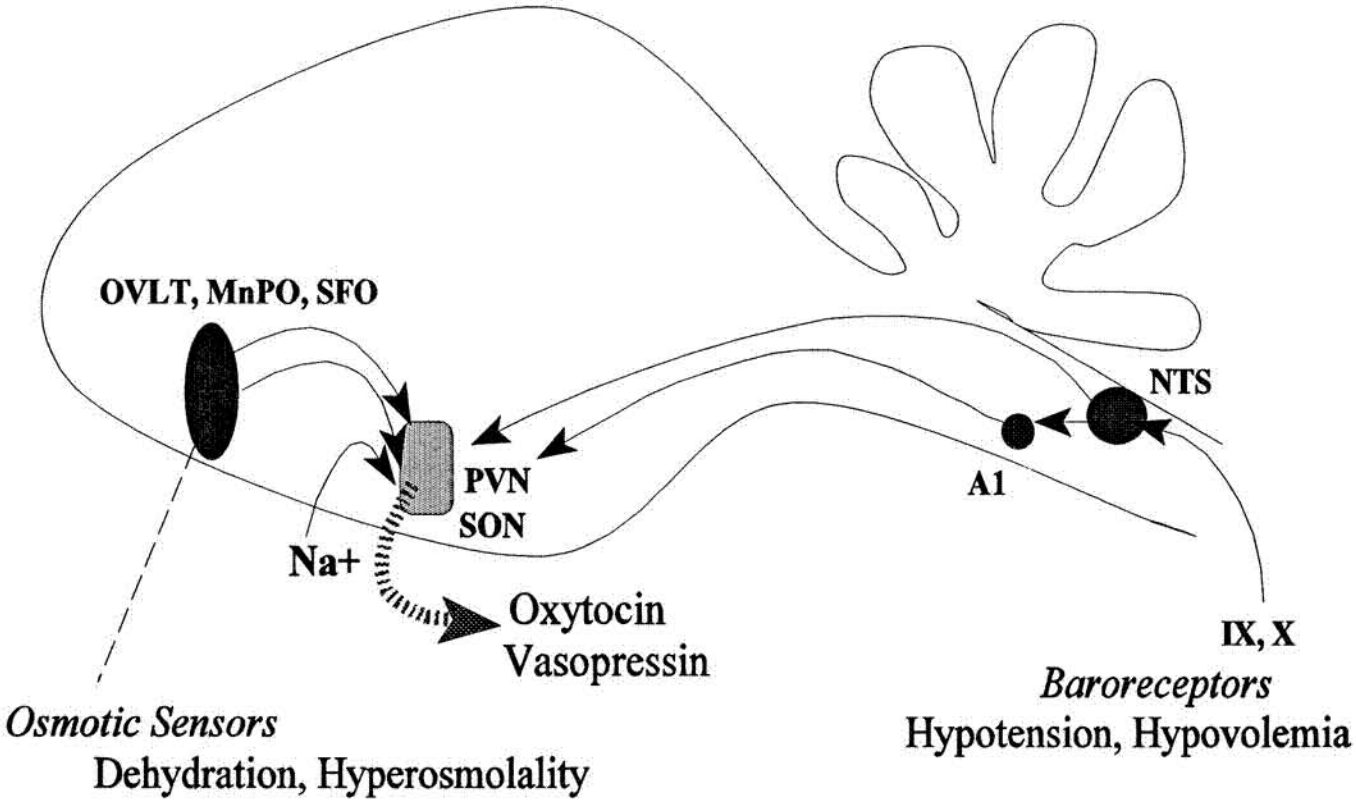
Higher mammals essentially regulate body water metabolism by monitoring total body water (via *volume-regulated* mechanisms) and body water osmolality (*osmolality-regulated* mechanisms: Berne & Levy, 1988). Body fluid osmolality is controlled by multiple homeostatic mechanisms that act to regulate body water by coordinating vasopressinergic (anti-diuretic hormone) levels and thirst. Osmoreceptors in the central nervous system can sense as little as a 2% increase in extracellular fluid osmolality and activate vasopressin release. This causes anti-diuresis and water conservation, resulting in increased fluid volume and decreased osmolality. Another hormone, angiotensin II, is also stimulated by plasma hyperosmolality and acts to stimulate thirst. Water ingestion results in restoration of fluid volume and normal plasma osmolality.

When body water is reduced by as little as 10%, volume-dependent mechanisms are activated. A fall in extracellular fluid volume (as occurs with water deprivation) is detected by baroreceptors, which stimulate the release of vasopressin (Figure. 2). A reduction in body water also lowers intra-arteriolar pressure within the kidney, resulting in an elevation in plasma angiotensin II concentration. This induces the organism to ingest fluids. Besides their anti-diuretic and dipsogenic (thirst-producing) effects, these hormones have other effects which help to maintain fluid balance. Both vasopressin and angiotensin II, for example, are potent vasoconstrictors that aid in maintaining arterial pressure during hypovolemia (Fitzsimmons et al., 1986).

The control mechanisms for body salt balance are inextricably related to factors that regulate body water (Reuss & Bello-Reuss, 1983). Acute intraperitoneal injections of hypertonic saline produces a decrease in blood volume by moving body water from the blood into the peritoneum, causing an elevation in plasma concentrations of angiotensin II and vasopressin (Hamamura et al., 1992). Disturbances of body fluid equilibrium are detected by central osmoreceptors and/or sodium receptors, and by peripheral vascular pressure receptors. Central osmoreceptors, lacking a blood brain barrier, are thought to reside in several forebrain regions (Figure. 2).

Figure. 2 Neural afferent systems controlling vasopressin and oxytocin secretion. Forebrain osmotic sensors in the SFO, OVLT and MnPO detect minute alterations in plasma osmolality (and/or sodium concentration) and control the firing pattern of antidiuretic hormone-containing neurons in the SON and PVN. This results in fine-tuned changes in blood concentrations of vasopressin, which controls water reabsorption by the kidney. Brainstem pathways, mediating blood pressure, also control the activity of SON and PVN neurons. Finally, recent work suggests SON and PVN neurons may be intrinsically sensitive to plasma sodium concentration (denoted by Na^+ in diagram). **SFO**, subfornical organ; **OVLT,** organum vasculosum of the lamina terminalis; **MnPO,** median preoptic area; **PVN**, paraventricular nucleus; **SON**, supraoptic nucleus; **DMN**, dorsal motor nucleus of the vagus nerve; **NTS**, nucleus of the solitary tract.

Neural Afferent Systems Controlling Vasopressin and Oxytocin Secretion



These brain sites include the subfornical organ (SFO), the organum vasculosum of the lamina terminalis (OVLT), and the median preoptic area (MnPO), are thought to operate as an *osmoreceptor complex* (Ferguson et al., 1986; Nunez et al., 1989; and Sladek et al., 1985). This complex is acutely sensitive to changes in circulating angiotensin II, which then induces animals to drink. Hypovolemia also induces drinking by the direct action of angiotensin II on this site. Information about vascular tone is transmitted by ascending neural pathways arising from visceroceptive regions of the brainstem. Neural inputs from the osmoreceptor complex and brainstem send direct projections to the neurohypophysial system. This input excites SON and PVN neurons to release vasopressin into the blood stream to modulate kidney water reabsorption. It is known that the SON and PVN are highly vascularized, and it is thought these regions may also be directly osmosensitive (Bourque, 1989; Mason, 1980). Recent work, then, suggests direct as well as indirect neural pathways (Leng et al., 1982, 1985, and 1989; Bourque, 1989) tonically mediate plasma vasopressin levels.

Similar to acute osmotic stimulation, long-term maintenance on hypertonic saline solutions induces a need to adjust body sodium and water levels. Previous research has described how this stimulus affects the structure and

function of the neurohypophysial system. Hypernatremia from salt water drinking elicits electrophysiological changes (Wakerly, 1982, 1987), neuronal hypertrophy, and activation of the expression of vasopressin and oxytocin genes in neurohypophysial neurons (McCabe et al., 1990; Sherman et al., 1986 and 1988; and Van Tol et al., 1987). This treatment also increases the levels of the mRNAs of several additional neuroendocrine substances in these neurons that are ordinarily at very low levels in these cells. Levels of the mRNAs for cholecystokinin (CCK), dynorphin, galanin, and the dopamine-synthesizing enzyme, tyrosine hydroxylase are dramatically increased by maintenance on salt water (Meister et al., 1990).

c-*fos* and c-*jun* Expression in the Neurohypophysial System

The expression of c-*fos* has proven to be an excellent tool for detecting increased intracellular activity in the CNS (Morgan et al., 1987; Morgan and Curran, 1991; Sagar et al., 1988b). Hypertonic saline injection causes a rapid and intense induction of c-*fos* expression in neurohypophysial neurons of the SON and PVN (Carter et al., 1990; Ceccatelli eta al., 1989; Ding et al., 1994; Giovannelli et al., 1990, 1992b; Guldenaar et al., 1992; Hamamura et al., 1992; Lafarga et al., 1992; Sagar et al., 1988a; Sharp et al.,

1991). Although the concentration or the amount of hypertonic saline administrated to animals may be different, it is generally reported that c-*fos* mRNA levels are at their highest 30 to 60 min after experimental treatment (Carter et al., 1990; Ding et al., 1994; Hamamura et al., 1992; Sharp et al., 1991). One report, using northern blotting, also indicates hypertonic saline injection induces c-*jun* mRNA expression (Carter et al., 1990).

The work outlined here is intended to extend our knowledge of c-*fos* and c-*jun* expression in the rat brain following hypertonic saline injection. I used a well-recognized experimental paradigm, acute injection of hypertonic saline, to stimulate hypothalamo-neurohypophysial neurons. I have focused primarily upon alterations of c-*fos* and c-*jun* expression in the magnocellular neurons of the supraoptic nucleus (SON) because a number of factors make this brain region an ideal model for studying stimulus-transcription coupling in neurons. Neurons of the SON form a homogeneous neuron population. The cells are contiguous, their activities of these neuron is controlled (at least partially) by their intrinsic sensitivity to changes in plasma osmolality, they form a homogeneous efferent neuronal system (since their terminals all project to the neural lobe of the pituitary gland), and metabolically they are very active neurons. My thesis attempts to answer four basic

questions. First, are c-Fos/c-*fos* and c-Jun/c-*jun* co-expressed in SON neurons after acute hypertonic saline injection? Second, if there is co-existent expression of these products, are there differences in the latency, duration, or intensity of expression of these two gene products? Third, is there overexpression of c-Fos/c-*fos* and c-Jun/c-*jun* following *repeated* hypertonic saline injection? Fourth, is there an increase in AP-1 DNA-protein binding activity in SON tissue after acute hypertonic saline injection?

Four different experimental techniques were used to answer the above questions: Double immunofluorescent methods, northern blotting, *in situ* hybridization, and gel shift DNA binding assay. Double immunofluorescent methods were used to determine if both Fos and Jun proteins appeared in the same SON neurons after saline injection and whether there were quantitative differences in the latency of appearance or duration of immunostaining for these products after saline administration. Also immunocytochemistry was used to determine whether there is a refractory period after hypertonic stimulation when Fos and/or Jun cannot be re-elicited by a second saline injection. Northern blotting and *in situ* hybridization techniques were used to determine the time course and anatomical pattern of expression of the mRNAs encoding c-*fos* and c-*jun* after hypertonic saline

administration. This work will indicate whether both mRNAs were expressed within the same time frame and at the same levels after saline injection, and how mRNA levels change after a second saline injection. The final series of experiments employed the gel shift DNA binding assay to verify that neuronal stimulation by hypertonic saline injection indeed results in the formation of AP-1 protein complexes capable of DNA binding activity. *In vivo* formation of the AP-1 complex in hypothalamic neurons will indicate there is actual protein complex formation in SON tissue that may allow for interaction with the AP-1 binding site consensus sequence.

CHAPTER 2

Colocalized Expression of Fos and Jun in Supraoptic Nuclei (SON) Neurons of Rats Following Fluid Imbalance

DOUBLE-LABEL IMMUNOCYTOCHEMICAL PROCEDURE FOR SIMULTANEOUS VISUALIZATION OF Fos AND Jun IN THE SAME NEURON

Introduction

Fos immunostaining can be induced in the central nervous system by a variety of stimuli. Depending upon whether the experimental treatment activates somatosensory, motor, neuroendocrine, autonomic, or emotional neural functions, the location of staining often occurs in different, physiologically-relevant, brain regions (Sagar, Sharp & Curran, 1988b; Krukoff, et al., 1994). The administration of hypertonic saline, for example, induces Fos immunostaining in regions known to control body water homeostasis, including the antidiuretic hormone-secreting neurons of the SON (Giovannelli et al., 1990, and 1992b; Sharp et al., 1991; Guldenaar et al., 1992; Hamamura et al., 1992; Lafarga et al., 1992; Ding et al., 1994). It has not

been determined, however, if the transcription factor, Jun, is expressed in brain areas that mediate water balance, or whether Fos and Jun co-expression occurs in the *same* neurons. As discussed previously, Fos is most effective as a transcription regulator when it forms a heterodimer with Jun, where the complex can then bind to sequence-specific regions of DNA and induce the expression of late-response genes. I have developed a double-label fluorescence immunocytochemical procedure that permits the simultaneous visualization of neurons expressing Fos and Jun. Determining that Jun expression occurs in the same cells that stain for Fos after osmotic stimulation will add significance to previous work, since it will provide evidence that the conditions exist for the formation of heterodimeric protein complexes that may then regulate gene transcription.

Materials and Methods

Experimental Animals

Eighteen adult male Long-Evans (Charles River Laboratories) rats, weighing 220-270 g, were housed under identical conditions and had free access to food and water. The rats were divided into three different treatment groups, each comprising six rats. Animals in Group 1 were not handled until they were anesthetized for sacrifice; animals in Group 2 were given an injection of isotonic saline (0.9% NaCl), and animals in Group 3 received an injection of hypertonic saline (1.5M/L NaCl). The salt solutions (0.018 ml/g body weight) were injected i.p. 90 min before the rats were anesthetized, and great care was taken to handle the rats identically. The rats were anesthetized with ketamine HCl (80 mg/kg, i.p.) and xylazine HCl (13 mg/kg, i.p.) and killed by decapitation. The brains were removed from the skull, blocked, and quickly frozen on dry ice. The blocked tissues were stored in liquid nitrogen (-196°C) until use.

Primary Antiserum

The species of origin and dilutions of the primary antisera were as follows: polyclonal rabbit anti-Jun/AP-1 (concentration of 2.5 μg/ml, Oncogene Science Inc., Uniondale, NY), polyclonal sheep anti-Fos (1:400, Cambridge Research Biochemicals, Inc., Wilmington, DE) in 0.1 M phosphate-buffered saline (PBS; pH 7.4).

To prove the specificity of the antibodies, tissue sections were processed with antisera that were preincubated with antigens. Anti-Fos serum was preabsorbed using 1.25 μg Fos peptide (Cambridge Research Biochemicals) per ml of 1:800 diluted serum. In accordance with the manufacturer's recommendations (Oncogene Science), 25 μg of Jun peptide was used to absorb 1ml of 1:80 diluted anti-Jun. The mixtures were incubated for 3 hr at room temperature with agitation. The aggregates were removed by centrifugation (5000 rpm for 10 min) and the supernatants were used for immunostaining.

Immunofluorescence

Ten μm cryostat sections were air-dried for 30 min at room temperature, fixed in cold acetone-methanol (50% v/v)

for 10 min at 4°C and rinsed three times in PBS. The sections were incubated with 10% normal donkey serum (Chemicon) in PBS for 15 min to block nonspecific binding, and then rinsed three times in PBS. Sections then were overlaid with one of the following mixtures of the two primary antisera (rabbit anti-Jun and sheep anti-Fos) that were incubated with: 1) neither Fos nor Jun, 2) both Fos and Jun, 3) Fos only, or 4) Jun only. Incubation with primary antisera was for 1 hr at room temperature by placing the slides in a moist chamber. The slides were rinsed three times for 30 min in PBS and then incubated for 30 min in a darkened room with a mixture of FITC-labeled donkey anti-sheep (1:20) and Rhodamine-labeled donkey anti-rabbit antibody (1:20) (Chemicon). After three rinses in PBS, the slides were coverslipped with 90% glycerol/PBS. Nuclear staining in SON tissue samples for Fos and Jun was observed under a Zeiss Axioskop microscope equipped with selective observation filters for FITC and Rhodamine. Images were photographed using the microscope's microprocessor-controlled camera and Ektachrome P800/1600 color reversal film.

Results

Ninety min after the administration of hypertonic saline, Fos and Jun immunoreactivity was present in SON neurons of Long-Evans rats (n=6: Figure 3 and Figure 4g,h). No signal was found in non-injected rats (n=6), and staining was seen in only a few SON neurons in tissue from isotonic saline injected (n=6) rats (data not shown). Absorption of antisera with both Fos and Jun (Figure 4a,b), either Jun (Figure 4c,d) or Fos (Figure 4e,f) resulted in the abolition of immunostaining for the corresponding antiserum.

Absorption of the Fos antibody with Jun and of the Jun antibody with Fos yielded no change in immunostaining of either antibody. The colocalization of Fos and Jun immunostaining was also detected in other regions of the hypothalamus, including the paraventricular nucleus (PVN), the nucleus circularis, the subfornical organ, the median preoptic nucleus (MnPO), and the organum vasculosum of lamina terminalis (OVLT: data not shown).

Figure 3. Co-localization of Fos [FITC-immunofluorescence; green color (**a**)] and Jun [rhodamine-immunofluorescence; red color (**b**)] nuclear immunostaining in the supraoptic nucleus of a male Long Evans rat, 90 min after an intraperitoneal injection of 1.5 M NaCl.

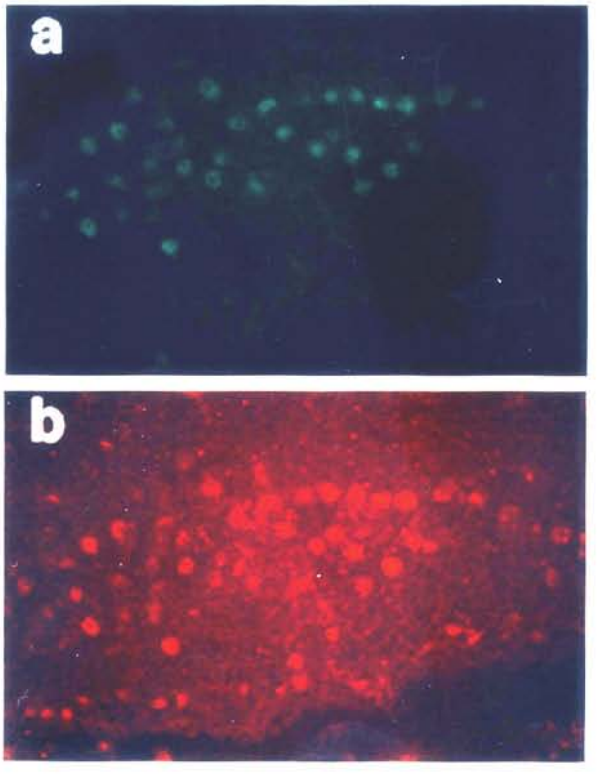
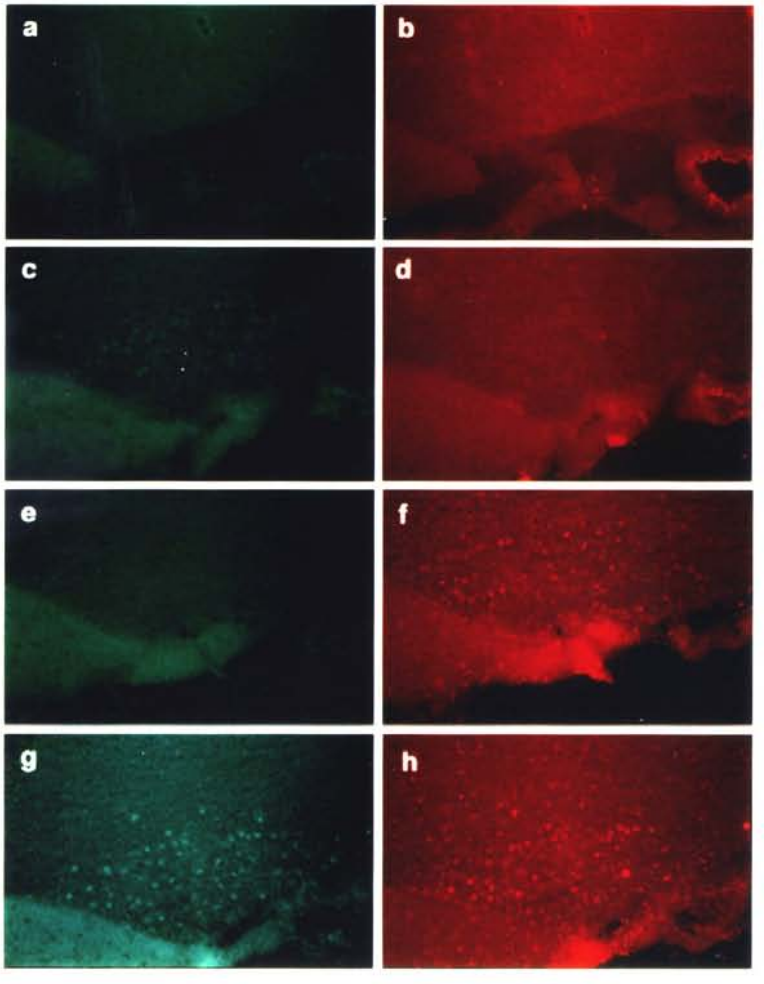

a
b

Figure 4. Nuclear immunostaining of Fos (greed color) and Jun (red color) in the supraoptic nucleus of a Long-Evans rat after acute hypertonic saline injection (1.5 M NaCl). (**a**) and (**b**), absorption of Fos and Jun antisera mixture with both Fos and Jun proteins. (**c**) and (**d**), absorption of Fos and Jun antisera mixture with only Jun protein. (**e**) and (**f**), absorption of Fos and Jun antisera mixture with only Fos protein. (**g**) and (**h**), no absorption of Fos and Jun antisera mixture with Fos or Jun protein.


a
b
c
d
e
f
g
h

Discussion

The administration of hypertonic saline induced the appearance of immunoreactivity for both Fos and Jun proteins in the same magnocellular neurons of the SON and PVN. The appearance of both proteins in the same cells is in line with molecular biological studies indicating the need for the cooperative expression of both Fos and Jun for there to be transcriptional activation of late response genes (Halazonetis et al., 1988; Nakabeppu et al., 1988).

Administration of hypertonic saline resulted in the appearance of Fos and Jun not only in magnocellular neurons of the SON and PVN, but also in other neurons, including the "osmoreceptive complex" (Honda et al., 1990): the subfornical organ (SFO), the median preoptic nucleus (MnPO), and the organum vasculosum of lamina terminalis (OVLT). These neurons integrate information about body fluid homeostasis and play a role in the control of magnocellular neuron activity (Chaudhry et al., 1989; Honda et al., 1990; Leng et al., 1982, and 1985; Sladek et al., 1985). These results agree with the previous work of Giovannelli (1992), as well as with the work of Hamamura and colleagues (1993) who broadly surveyed the CNS and found Fos immunostaining in the osmoreceptive complex, SON, and the PVN.

Fos and Jun immunostaining was essentially absent in non-injected rats. This finding agrees with other reports, which examined tissue processed for Fos staining by sensitive immunoperoxidase methods (Giovannelli et al., 1990; Guldenaar et al., 1992). In regions other than the SON, a few scattered Fos and Jun immunostained nuclei were found in the tissue samples from isotonic saline-injected rats. These data are in agreement with the findings of Giovannelli and colleagues (1990), but are different from other earlier reports where Fos staining was seen in many brain regions (Dragunow and Faull, 1989; Guldenaar et al., 1992). The latter reports suggested some Fos immunostaining is seen in the SON, and substantially more staining in other brain areas, as a result of the stress of handling by the investigator and the experience of receiving an isotonic saline injection. In fact, these workers reported a dramatic dichotomy in immunostaining between rats that were not handled and those that receive an isotonic injection. Guldenaar and coworkers (1992), for example, found handled rats invariably had Fos immunostaining in limbic system brain areas; systems known to mediate stress and emotional-behavior response patterns. In the present work, I have used a less sensitive technique, employing immunofluorescent markers as second antibodies, instead of peroxidase labelled antibodies. It is generally agreed that immunofluorescent

methods have a relatively lower sensitivity.

The serum specificity of the antibodies was corroborated by absorption of a mixture of anti-Fos and anti-Jun serum with either Fos or Jun peptide. In the former case, only Jun immunostaining was found, whereas all Fos was abolished. In the second case, Fos but not Jun immunostaining was detected. When a mixture of anti-Fos and anti-Jun serum were absorbed with both Fos and Jun peptides, no staining for either antigen was found.

In conclusion, a double-label immunofluorescence method was employed to determine if hypertonic saline administration could induce Fos and Jun staining in brain regions known to play a role in the mediation of body water balance. This treatment resulted in the majority of neurons exhibiting immunostaining for both antigens, This result is important because it shows: 1) the hypertonic stimulus is able to induce Jun staining, 2) the staining for Jun occurs in neuron cell groups (SON, PVN, OVLT, SFO, MnPO) which are known to play a role in body water balance, 3) the majority of neurons exhibit coordinated expression for both Fos and Jun, and 4) since Fos must form a heterodimeric complex with Jun to control transcription, it further reinforces the physiological significance of previously reported observations of Fos immunostaining in specific regions.

TIME COURSE OF COLOCALIZED c-Fos AND Jun EXPRESSION IN SON AFTER ACUTE FLUID IMBALANCE

Introduction

In the previous section, I reported c-Fos and c-Jun immunostaining co-exists in the same SON neurons 90 min after hypertonic saline injection. A time-course study was next undertaken to determine the latency of appearance and duration of immunostaining for both Fos and Jun. In 1991, Sharp and colleagues found that Fos protein, detected using a specific monoclonal antibody, was maximal 1-2 hr after saline injection and disappeared 4-8 hr later. Fos-like immunoreactivity detected using two polyclonal antisera, however, was observed in SON for 7 days. So far, no group has determined the temporal changes in c-Jun expression in SON following osmotic stimulation, or whether the temporal expression for Fos and Jun are similar. Using the double-immunofluorescence method outlined earlier, I determined the pattern of Fos and Jun immunoreactivity in the SON 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 24 hr after injection of hypertonic saline.

Materials and Methods

Experimental Animals

Male Long-Evans rats (200-300 g) were purchased from Charles River Labs. and housed individually (12 hr light/dark cycle) with access to food and water. A total of 51 rats received a hypertonic saline (1.5 M/L NaCl) injection. Five additional rats served as a control group and received no treatment. No normal saline (0.9 % NaCl) treated rats were included in this study since the results of the last experiment indicated this treatment does not induce Fos or Jun immunostaining. Immediately or 0.5, 1, 1.5, 2, 3, 4, 6, 8 or 24 hr after injection, the rats were anesthetized with Ketaset (80 mg/Kg) and Rompun (13 mg/Kg) administered i.p. When unresponsive to paw pinch, they were decapitated by guillotine, and the brain was removed from the calvarium, blocked with a clean razor blade, and frozen in liquid nitrogen. At the time of decapitation, trunk blood was collected and stored in a refrigerator (4°C) until it was analyzed for osmolality, Na^+, Cl^-, and K^+ content. Plasma measurements were performed by the Clinical Pathology Laboratory, USUHS, by using a Kodak Ektachem 700 Analyzer (Kodak, Rochester, NY).

Immunocytochemistry Protocol

Tissue containing the SON was processed as described above to determine the time course of the colocalized appearance of Fos and Jun in SON neurons.

Data Analysis

For assessment of the incidence of immunostaining of SON neurons, slides containing SON tissue were observed using a Zeiss Axioskop microscope equipped with filters for viewing material labeled with FITC and Rhodamine. An attached 35mm camera allowed one to photograph tissue fields, and this was performed with Ektachrome P800/1600 color reversal film. A minimum of 3 slides were photographed from each rat SON. A slide projector was used to project photographic slide images of the SON on a wall. The middle region of each section of the SON (where the height and breadth of the SON is greatest) was selected for recording the number of positive immunostained neuron nuclei. For each rat, a record was kept of the number of nuclei positively stained for Fos, Jun, or both antigens, as well as unlabeled SON neurons. These counts were used to determine the percentage of neurons stained for one,

neither, or both antigens (Guldenaar, et al., 1994). Tissue sections varied between experiments in their level of background staining. The level of background staining seen in tissue from control rats was used to assess background in each experiment.

A single-factor analysis of variance was used to evaluate statistical significance, by employing the SigmaStat software program (version 1.01, Jandel Scientific, San Rafael, CA). Bonferroni's test was used when overall significance was indicated, where groups were compared with the data from the control group. Data were summarized using SigmaPlot (version 5.0, Jandel Scientific) and expressed as the mean $\pm$ the standard error of the mean.

Results

Based upon the results obtained in initial experiments, I found there was no detectable Fos or Jun immunostaining of SON neurons in rats that were sacrificed 15 min after saline injection (n=3) as well as 6, 8 and 24 hrs postinjection (n=2 rats per group). In order to use less animals and reagents, these time points were eliminated from the experiment and no further animals were sacrificed for these time points. This reduced the number of experimental groups for histological analysis from 12 to 8.

Thirty minutes after saline injection, plasma osmolality and sodium concentration were increased above baseline levels in rats that had received no injection (labeled **Co** in Figure 5A and B). In rats that were killed immediately (<2 min, labeled **0.0**) after hypertonic saline administration, osmolality levels were equivalent to the noninjected group. Plasma osmolality and sodium levels of rats that were sacrificed later than 90 minutes after saline injection were at baseline levels (Bonferroni's test: $p > 0.05$).

In animals that were sacrificed without any hypertonic saline injection (**Co** group), or that were killed immediately

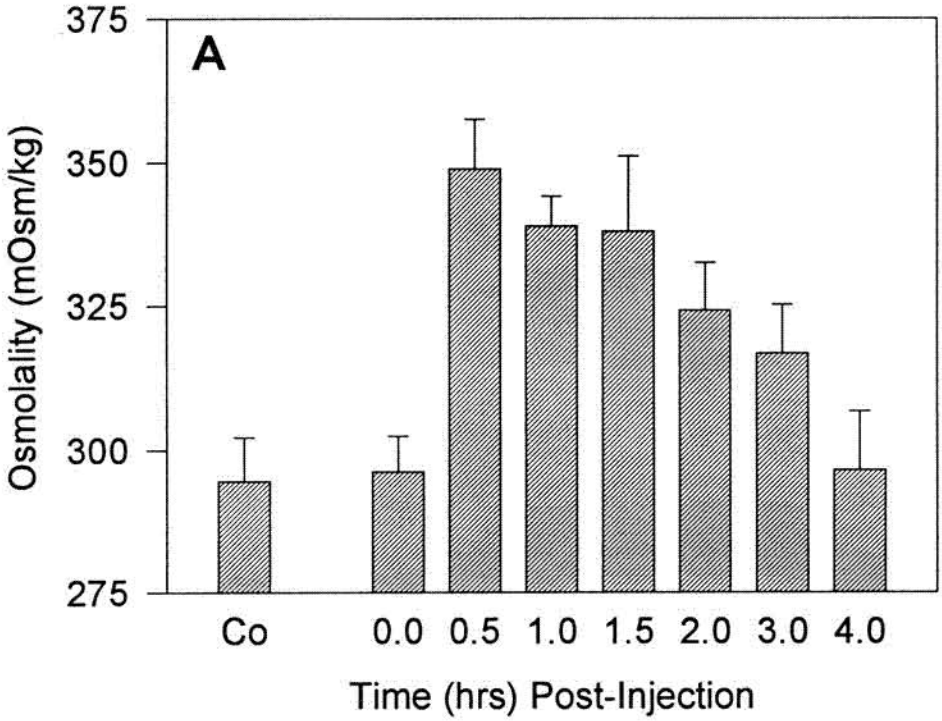
(<2 min) after injection (**0.0** group), a few SON neurons were found to stain for Fos and Jun (Figure 6). Thirty min after injection (**0.5** group), there was a significant increase in the number of cells immunostaining for both antigens (~20%) as well as in the amount of immunoreactivity of both antigens per cell for both proteins, the percentage of neurons exhibiting immunostaining, as well as the magnitude of immunoreactivity per cell was maximal at 1.5 hrs and 2 hrs, with over 80% of SON neurons possessing Fos and Jun immunostaining. By 4 hr after injection, less than 20% of SON neurons exhibited immunostaining to Fos and Jun, and the intensity of antigen immunoreactivity per cell was decreased. (Figure 6A)

Co-localization of Fos and Jun immunostaining was not observed in every SON neuron. A few SON neurons appeared to express either Fos or Jun immunostaining. Figure 6B summarizes analyses of the percentage of neurons that exhibited immunostaining to one, but not both, Fos and Jun. The number of singly-labeled neurons was never more than 5% for either antigen. There appeared to be some difference, however, in the percentage of cells that labeled for Fos or Jun as a function of time. By 3 hr after saline injection, more of the cells immunostained for Jun (p <0.05 compared to levels in the **Co** group) than for Fos (**Co** *vs.* 3 hr group, p >0.05). It is noted that singly-labeled cells comprise a

very small percentage of SON neurons: however, it may be a substantial number of cells considering there are approximately 4,500 neurons per SON (about 225 neurons).

Figures 5A and B summarize plasma osmolality and sodium levels of rats sacrificed at different time points after injection of hypertonic saline. **Co** on the abscissa refers to data from rats that received no injection (control), while the zero time point (**0.0**) refers to rats that received an injection followed by immediate sacrifice. The error bars indicate the standard error of the mean. Rats sacrificed 30, 60 and 90 minutes after injection exhibited blood osmolality (A) and plasma sodium values (B) that were significantly greater than the control group (all $p < 0.05$), while osmolality values for rats sacrificed at the 0.0 time point, or 2, 3, and 4 hr post-injection were not different from the non-injected control group.

Time Course Study of Plasma Osmolality (A) and Sodium Concentration (B) After Hypertonic Saline Injection



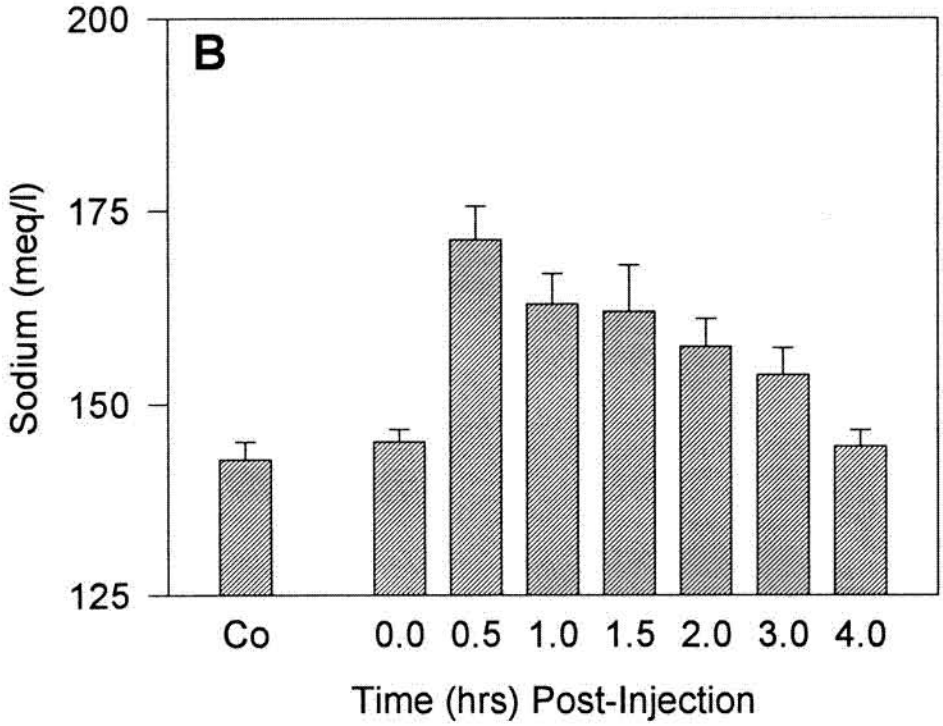
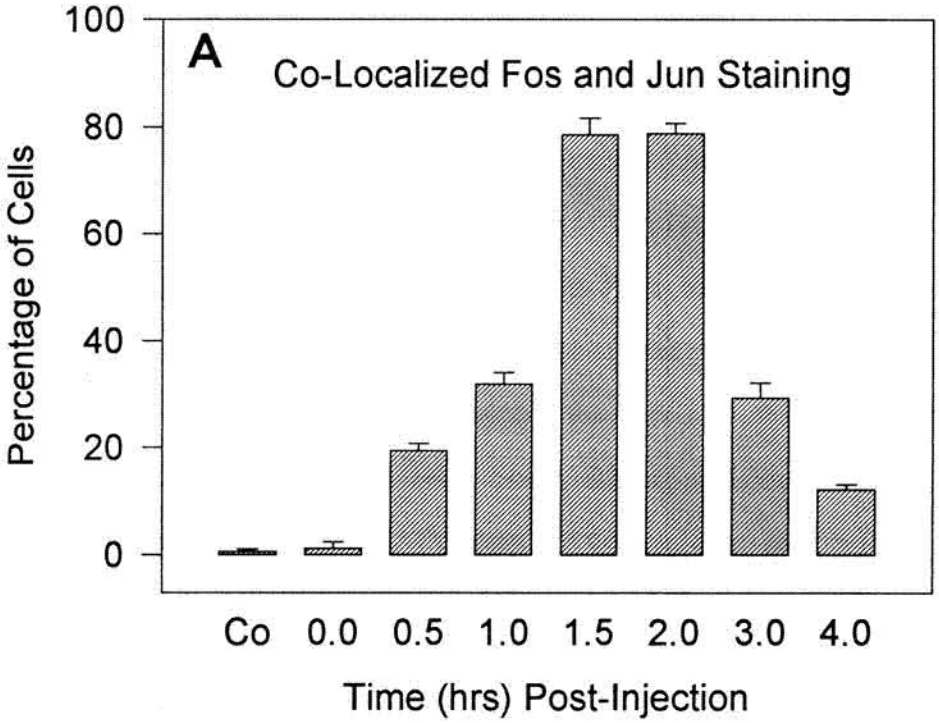
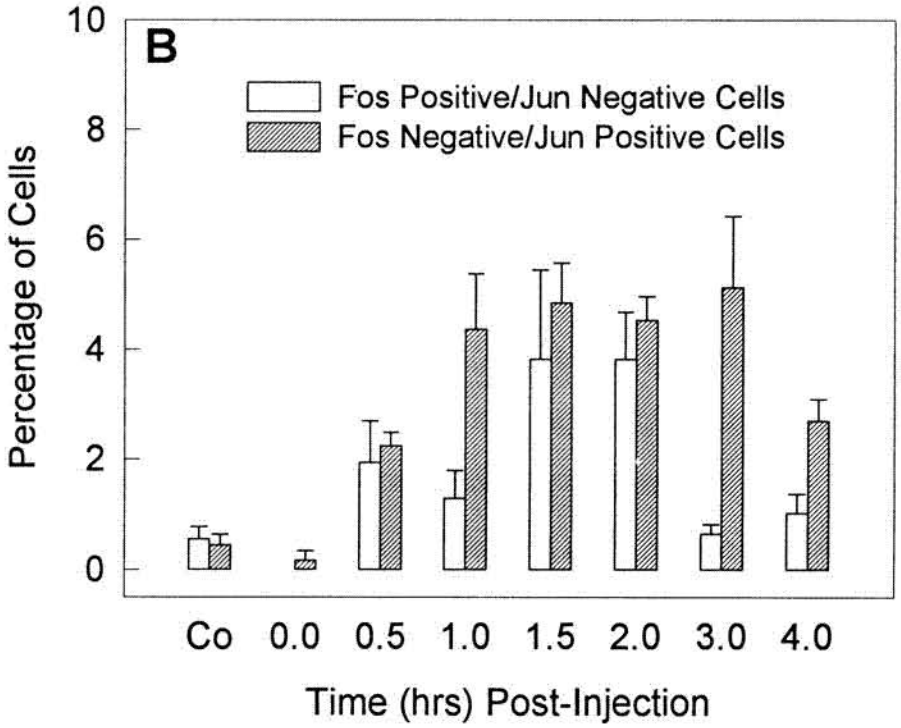


Figure 6. summarizes data obtained from tissue samples of rats sacrificed at the various chosen time points after hypertonic saline administration. The top bar graph (**A**) summarizes analyses of the percentage of neurons that exhibited immunostaining to both Fos and Jun. With the exception of the **0.0** time group, the percentage of neurons expressing Fos and Jun at all time points was significantly greater than levels seen in rats that received no injection (**Co**).

The lower bar graph (**B**) summarizes analyses of the percentage of neurons that exhibited immunostaining to one, but not both, Fos and Jun. By 90 min and 2 hr after injection, the percentage of neurons with Fos immunostaining (Fos Positive/Jun Negative Cells) was greater than levels seen in the non-injected rats (**Co**). At the time points 1, 1.5, 2, and 3 hr post-injection, the percentage of cells positive for Jun was greater than the **Co** group.

Percentage of SON Neurons With Immunostaining for Fos and/or Jun





Discussion

This experiment assessed the duration and intensity of co-localized expression of Fos and Jun in magnocellular neurons of the SON following hypertonic saline administration. Within 30 minutes, intraperitoneal administration of hypertonic saline had induced both Fos and Jun immunostaining in 20% of SON neurons (Figure 6A). Levels of immunoreactivity peaked 90 to 120 min later (more than 80% of SON neurons), and then disappeared by 4 hr (less than 20% of SON neurons). These findings agree with the work of Sharp and colleagues (1991), who found, using a monoclonal antibody (LA041) that was raised against the c-Fos N-terminus, that Fos protein is induced within a time course similar to what was used here, and that immunostaining had disappeared within 4-8 hr. They also have reported that the osmotically induced expression of Fos-related antigens (FRA), detected by an antibody recognizing the M peptide (Fos 127-152), endures for as long as 7 days. The antibody that I used in these studies (a sheep polyclonal antibody, Cambridge Res. Biochem.), had been raised against the N-terminal portion of the c-Fos protein, so that the degree of homology between c-Fos and Fos-related antigens is reduced. However, I cannot exclude the possibility that the antibody I used may also recognize

FosB or other proteins whose N-terminus may have a similarity to c-FOS (Zerial et al. 1989). The present work does appear to be in line with Sharp's work with a monoclonal antibody.

It was apparent from my results that not all magnocellular neurons had co-existent Fos and Jun immunostaining: a few SON neurons only expressed c-Fos or c-Jun protein. In this group of neurons, the percentage of cells, as well as the duration of expression, was greater for c-Jun than levels of c-Fos. This dissociation may be due to one or all of the following factors. 1) It is generally believed that SON neurons are a single, homogenous population of cells; identical in their morphology and chemical characteristics, as well as the nature of their neuronal inputs (for review see Swanson et al. 1983). However, these neurons may be more heterogenous than previously suspected (Roberts et al. 1993). The appearance and duration of Fos or Jun immunostaining in each single neuron may be slightly different (Herdegen et al. 1991a and 1991b; Gass et al. 1992). 2) Since members of the Jun family, c-Jun, Jun B, and Jun D, can form homodimers or heterodimers with each other, they may have very different effects upon gene expression (Mellström et al., 1991). Hypertonic saline injections may induce magnocellular neurons to not only express c-Jun but also Jun B and/or Jun

D and it may be these other factors affect the concentration of c-Fos and c-Jun. 3) Jun family members are not the **only** transcription factors which physically interact with Fos. Using the c-Fos basic leucine-zipper dimerization motif as a molecular probe to screen a human cDNA library, Blanar and Rutter (1992) have isolated a clone encoding a member of the basic helix-loop-helix-zipper (bHLH-Zip) family of proteins. This protein is called FIP (for Fos Interacting Protein). Fos can exert transcriptional activity in cooperation with FIP. However, the interaction of FOS-FIP complexes with DNA is not yet clear (see Blanar and Rutter, 1992). 4) Finally, I must consider the level of sensitivity of the fluorescence immunocytochemical method. It may be that more cells contain Fos and Jun protein, but that the concentrations are below the sensitivity of this technique.

COLOCALIZED c-Fos AND c-Jun EXPRESSION IN SON NEURONS FOLLOWING *REPEATED* FLUID IMBALANCE

Introduction

The final immunostaining experiment examined the nature of the disappearance of Fos and Jun immunostaining after osmotic stimulation. In the previous experiment, I observed that 4 hr after a hypertonic saline injection Fos and Jun immunostaining had almost disappeared. This experiment was conducted to see if at the point when Fos and Jun immunostaining has almost disappeared (4 hr post-injection), one can re-elicit immunostaining by a second administration of hypertonic saline.

Materials and Methods

Experimental Animals

Twenty-five male, Long-Evans rats were randomly assigned to receive one of following treatments; the procedure is summarized in the Table 1.

The rats in Group 1 received a 1.5 M injection of saline, were returned to their cage for 4 hr, and then received a second hypertonic saline injection. These rats were then sacrificed 90 min later. Group 2 received a similar treatment, except the two injections were 0.9% saline. Group 3 first received 0.9% saline, and then a 1.5 M hypertonic injection. Group 4 was used as positive control group, receiving a single 1.5 M hypertonic injection followed by a 90-min survival time. Group 5 received two hypertonic saline injections, but were killed 30 min after the second injection. The purpose of this group was to verify that a second injection of hypertonic saline is able to elicit a robust change in plasma osmolality and sodium content. All rats were sacrificed using the procedure described earlier.

Table 1

Summary of Injection Scheme and Time Course for Treatment Groups

Group (n=5)	1st Injection	2nd Injection	Survival Time
1	1.5 M NaCl	1.5 M NaCl	4 hr + 90 min
2	0.9% NaCl	0.9 % NaCl	4 hr + 90 min
3	0.9% NaCl	1.5 M NaCl	4 hr + 90 min
4	1.5 M NaCl	____	90 min
5	1.5 M NaCl	1.5 M NaCl	4 hr + 30 min

Immunocytochemistry Protocol

The double-label immunocytochemical procedure was identical to the method outlined earlier.

Data Analysis

A single-factor analysis of variance was performed as described earlier. Bonferroni's method for pairwise multiple comparisons was used here, however, to assess differences between data collected from all treatment groups.

Results

All rats that received a hypertonic saline injection exhibited a significant increase in osmolality (Figure 7A) and sodium concentration (Figure 7B) that was greater than levels seen in rats that received two normal saline injections (**2xNS-90'** group). The rats that received two hypertonic saline injections and were sacrificed 30 min after the second injection (**2xHS-30'**) exhibited higher osmolality and sodium levels than all other groups. All groups of animals sacrificed 90 minutes after an injection, regardless of whether they had received just one hypertonic saline injection (**1xHS-90'** and **NS-HS-90'** groups) or two hypertonic saline injections (**2xHS-90'**), had equivalent osmolality and sodium values.

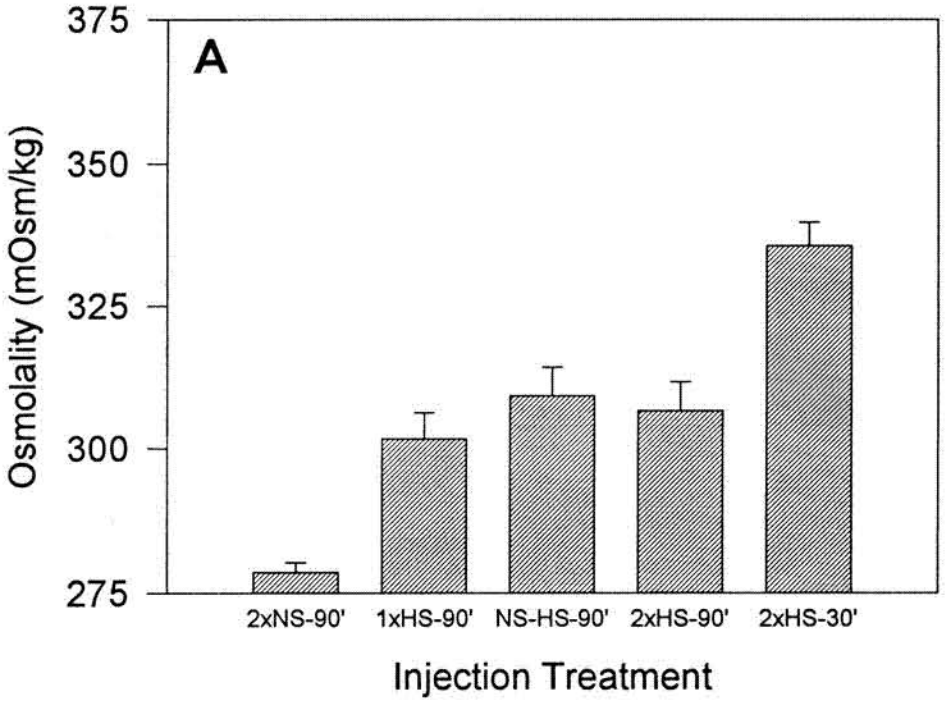
The rats that were injected twice with hypertonic saline (Figure 8A, labeled, **2xHS-90**) exhibited an increase in colocalized immunostaining, compared with rats that were twice injected with normal saline (**2xNS-90**). The increase after *two* injections (<20% colocalized staining for Fos and Jun), however, was dramatically different from what was seen after a *single* hypertonic saline injection (either **1xHS-90** or **NS-HS-90**), where 90 minutes after hypertonic saline administration greater than 80% of the neurons of the SON were labeled with

both antigens. Colocalized immunostaining of Fos and Jun was the same in rats that either received a single hypertonic saline injection (**1xHS-90**) or a normal saline and followed by a hypertonic saline injection (**NS-HS-90**; see Figure 8A).

SON neurons from rats injected with normal saline twice (**2xNS-90** in Figure 8B) had few cells labeled for Fos or Jun. In contrast, after two hypertonic saline injections (**2xHS-90**), a low but significantly large percentage of cells expressed Fos immunoreactivity (20%). There were few cells immunostained for Jun regardless of the experimental treatment (Figure 8B, Fos Negative/Jun Positive Cells).

Figures 7A and B summarize measurements of plasma osmolality and sodium concentration, respectively, in the five groups of animals in this experiment. One group of rats received *two* normal saline injections (labeled, **2xNS-90'**), a second group received a *single* injection of hypertonic saline (**1xHS-90'**); a third group first received a normal saline injection and then a hypertonic saline injection (**NS-HS-90'**); another group of rats received two hypertonic saline injections (**2xHS-90'**), and a fifth group of rats received two hypertonic saline injections, 4 hrs apart, but they were sacrificed 30 min after the second injection (**2xHS-30'**). Osmolarity and sodium measures for all groups were statistically greater than the **2xNS-90'** group. Multiple comparisons indicate the plasma values for rats that received two hypertonic saline injections and were sacrificed 30 minutes after the second injection had significantly greater mean osmolality and sodium values, while rats that were sacrificed 90 minutes after a hypertonic saline injection (whether or not it was preceded by another injection) were not statistically different ($p > 0.05$).

Plasma Osmolality (A) and Sodium Concentration (B) After Saline Injection Treatment



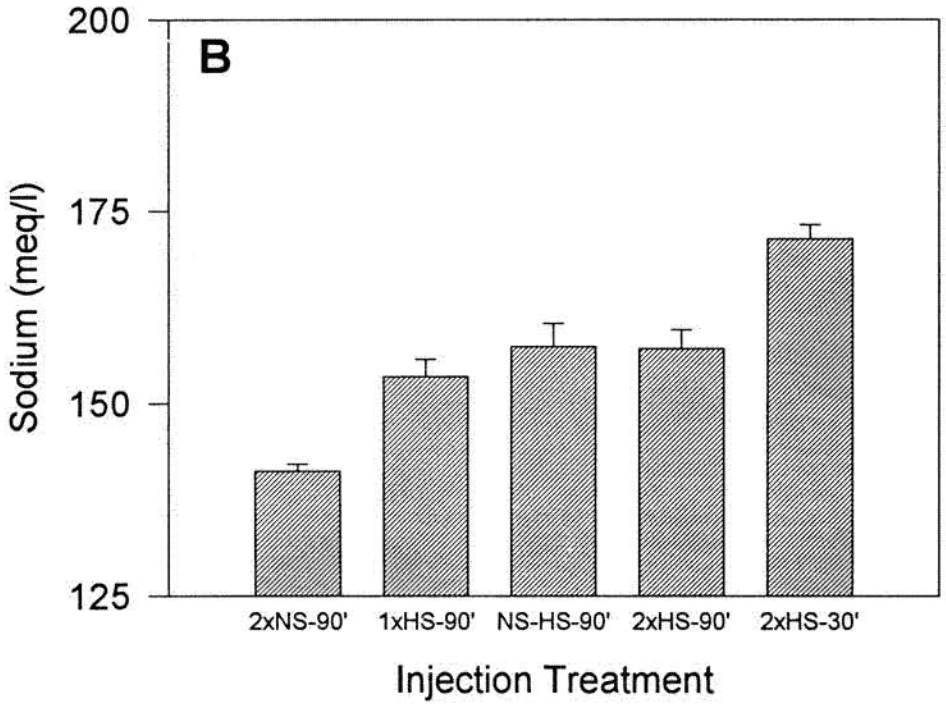
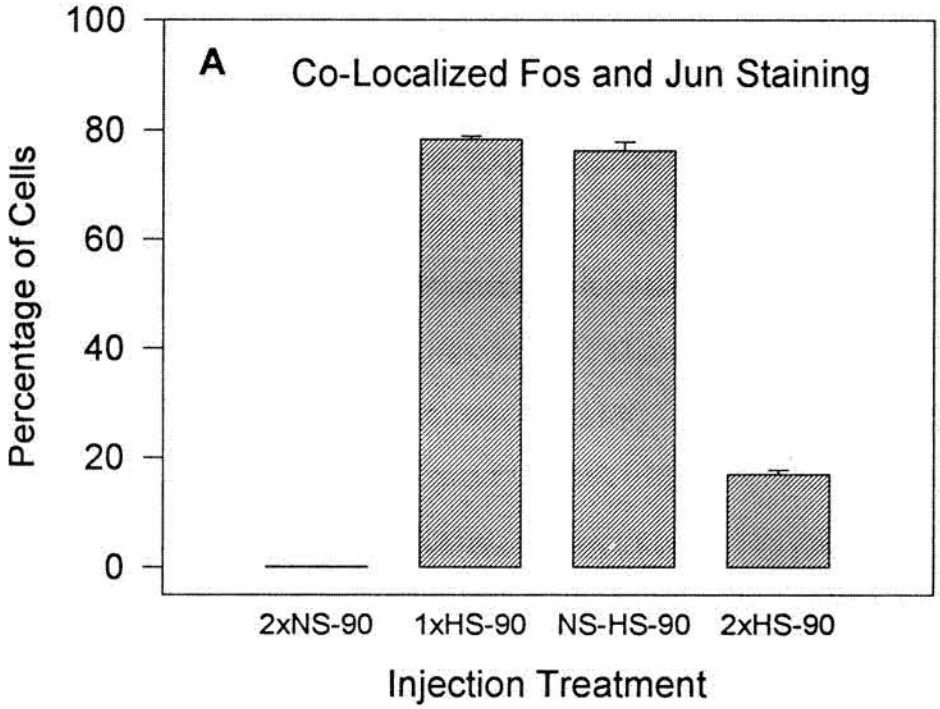
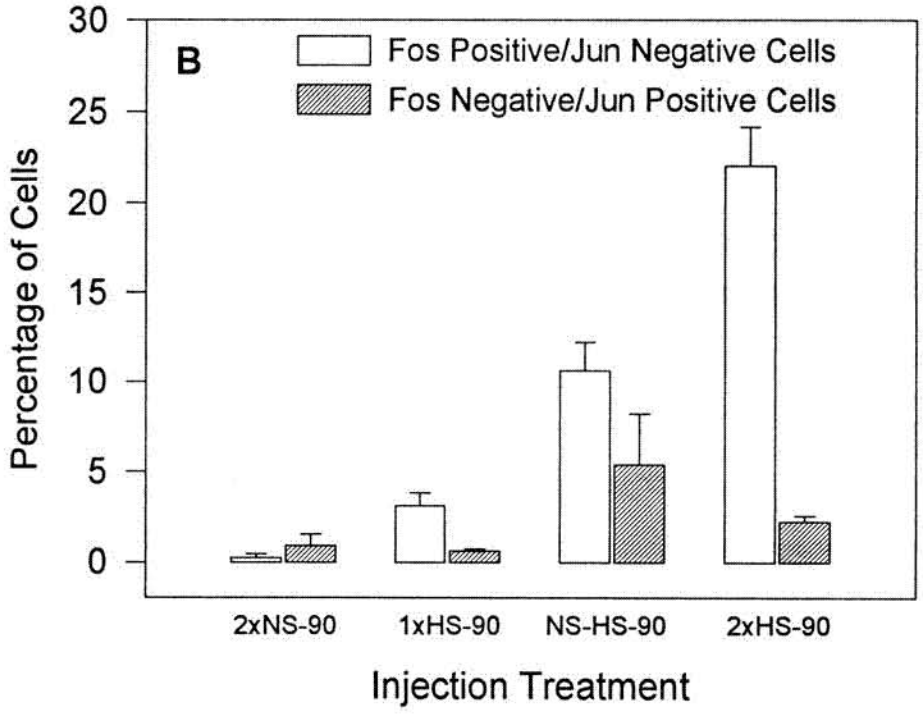


Figure 8 summarizes the percentage of SON neurons that had colocalized expression of both Fos and Jun (**A**), or (**B**) immunostaining for either Fos or Jun, after acute or repeated hypertonic saline injection. In tissue from rats that received single injections of hypertonic saline (**1xHS-90** and **NS-HS-90**), colocalized expression (**A**) was greater than 80%, and these values were statistically greater than the mean percentage for rats that received two normal saline injections (**2xNS-90**). The mean percentage of co-labeled neurons in SON tissue from rats that received two hypertonic saline injections (16.9%: **2xHS-90** group) was significantly less than the groups that received just one hypertonic saline injection, but it was significantly greater than that the **2xNS-90** group.

The percentage of cells singly-labeled for Fos (Figure B: Fos Positive/Jun Negative Cells) in rats receiving two normal saline injections (**2xNS-90**) or one hypertonic saline injection (**1xHS-90**) was less than levels seen in either of the other groups of rats that received one (**NS-HS-90**) or two (**2xHS-90**) hypertonic saline injections. The percentage of neurons labeled by the Fos antibody for rats that received two hypertonic saline injections (**2xHS-90**: 22.1%), was substantially greater than all other groups ($p < 0.05$). With respect to Jun immunostaining, analysis of variance indicated all groups were equivalent ($p = 0.0681$).

Percentage of SON Neurons With Immunostaining for Fos and/or Jun





Discussion

The percentage of SON neurons with co-localized Fos-Jun immunostaining was dramatically different in rats that received a single or a double injection of hypertonic saline. Similar to the levels seen in Experiment 2, a single injection of hypertonic saline, whether it was or was not preceded by a normal saline injection (Figure 8A, **NS-HS-90** and **1xHS-90**, respectively), resulted in greater than 80% of SON neurons having co-localized immunoreactivity. In contrast, co-localized expression was significantly less (<20%) when rats received two hypertonic saline injections (Figure 8A, **2xHS-90**).

The percentage of singly-labeled neurons after one or two injections was different for Fos and for Jun (Figure 8B). There was no significant increase in the percentage of singly-labeled Jun-positive neurons after two hypertonic saline injections, while the percentage of Fos-positive (Jun-negative) cell nuclei was considerably greater in rats that received two hypertonic saline injections. These results, when also considered in light of the percentage of double-labeled cells, suggest the total percentage of Jun-positive neurons after two injections is less than the percentage for Fos-positive cells. The total percentage of nuclei immunostained for Fos was 39% if one considers both double-

labeled (16.9%) and singly-labeled cells (22.1%) collectively, and the percentage of Jun-positive cells was just 19% of SON neurons (16.9% + 2%). Compared to values observed in the case of tissue from rats that received single hypertonic saline injections, where the total percentage of neurons staining for Fos was 82.4% of the neurons (**1xHS-90** and **NS-HS-90** groups, respectively), then, the percentage of Fos immunostained neurons was about half for the group that received two hypertonic saline injections (39%). On the other hand, the total percentage of neurons immunostaining for Jun was about one-quarter of SON neurons for the group of rats sustaining two hypertonic saline injections (19.2%) compared to values observed in the case of tissue from rats that received single hypertonic saline injections, where the total percentage of neurons staining for Jun was 81.6% of the neurons (**1xHS-90** and **NS-HS-90** groups, respectively).

While the present work does not determine the underlying causes for less immunoreactivity after repeated injections, there are data from other cell models that may be relevant. It is known that c-Fos is autoregulated (Sassone-Corsi et al., 1988). In co-transection assays, for example, the constitutive expression of c-Fos suppresses c-*fos* promoter activity by interaction with multiple sequences in the 5' flanking region (Treisman et al., 1985; Sassone-Corsi et al., 1988; König et al., 1989; Lucibello et al., 1989; Schönthal et

al., 1989), and the association of c-Fos with Jun enhances transrepression by c-Fos (Lucibello et al., 1989; Schönthal et al., 1989). Two AP-1-like sequences, positioned -60 and -296 upstream of the initiation site, may be involved in c-*fos* gene down-regulation through the binding of c-Fos/c-Jun complexes (Schönthal et al., 1989). Finally, the SRE (serum response element), located 300 bp upstream from the cap site in the 5'-flanking portion of c-*fos* gene, is known to often be the main target for repression (König et al., 1989; Lucibello et al., 1989; Schönthal et al., 1989; Subramaniam et al., 1989).

If rats were exposed to repeated hypertonic saline stimulation, c-Jun expression was greatly diminished. No reports have delineated the processes that may account for this observation, but again *in vitro* work may be relevant. Using F9 and F9-B1-C cell culture Chiu et al. (1989) demonstrated that Jun-B can down-regulate c-Jun translation. The same authors reported that c-Jun is an efficient activator of c-Jun and collagenase promoters which contain a single TRE. However, Jun-B efficiently inhibited c-Jun stimulation of these genes. Schütte and coworkers (1989) also have shown that Jun-B inhibits, and c-Fos stimulates, the transactivating and transforming potential of c-Jun in F9 cells and murine fibroblasts, respectively.

I cannot exclude the possibility that acute and repeated

hypertonic saline injections also induce the expression of other relevant members of the Fos and Jun families, such as Fos B, Fra 1, Fra 2, Jun-B and Jun-D. These factors are known to exhibit different responses, depending upon the stimulus used (Herdegen et al., 1991a and 1991b; Gass et al., 1992), and it may be they play a role in causing different response patterns after repeated stimulation. It is possible, for example, that following repeated osmotic stimulus, c-Fos forms heterodimers with other members of the Jun family that stimulates the transcription of some genes while inhibiting the expression of others. With respect to what the roles are for Fos and Jun in the control of vasopressin and oxytocin expression, the present work does not address whether a single stimulus (or repeated stimulation) results in changes in gene transcription. I also cannot exclude the possibility that acute and repeated hypertonic saline injections also induce expression of other members of Fos and Jun families, such as Fos B, fra 1, fra 2, Jun-B and Jun-D, that in turn have dramatic effects upon hormone synthesis.

How one should view hypertonic saline as an experimental treatment is also a complex issue. Hypertonic saline administration has at least three effects upon an animal: it is an osmotic stimulus (dramatically changing blood osmolality), the injection itself causes tissue damage (albeit minimal), and it is at least a mildly stressful experience for

the rat. I attempted to study the impact of the stress of handling the animal, and the effect of repeated tissue penetration with the needle, by including rats that received injections of normal saline. Repeated handling, tissue damage from intraperitoneal penetration, and two doses of normal saline (**2xNS-90'**) did not significantly increase staining (Figure 8A), and levels were essentially equivalent to those seen in rats that received no injection or were sacrificed after a single hypertonic saline injection (**Co** and **0.0** groups, respectively in Figure 6A). Likewise, the impact of the repeated handling and tissue damage experience in rats that received one hypertonic saline injection preceded by normal saline administration (**NS-HS-90**) was equivalent to results seen after a single hypertonic saline injection experience (**1xHS-90**). These results suggest one can discount that the differences in the percentage of immunoreactive SON neurons for the groups receiving one (**1xHS-90**) or two (**2xHS-90**) injections is due to tissue damage or handling effects and that for this experimental paradigm hypertonicity is the predominant factor altering SON immunoreactivity.

With respect to the ability of repeated saline injections to alter blood values, a group of rats were also sacrificed 30 minutes after receiving a second hypertonic saline injection. This group was included to test whether the hypertonic saline treatment condition causes a substantial change in plasma

osmolarity (Figure 7A) and sodium concentration (Figure 7B) akin to what is observed after a single hypertonic saline injection (Figures 5A and B, respectively). It was an important test for assessing the effectiveness of the hypertonic saline treatment. For example, any decrement in colocalized immunoreactivity in tissue from rats that received two hypertonic saline injections could have been due to a (unknown) homeostatic process initiated and still active as a consequence of the first hypertonic saline injection.

In view of attempts to ensure the equivalency of the degree of stimulation across experimental groups, two hypertonic saline injections may still be seen as two stressful and painful experiences. From this view, it is still impressive that two injections of hypertonic saline led to no equivalent (or greater) percentage of immunostained neurons. Most research indicates that (except for very long term stimulation) repeated stress upregulates or at least leads to a second response of the neuroendocrine system in terms of the release or production of neuroactive substances (Irwin et al., 1986; Adell et al., 1988; Nisenbaum et al., 1991). Chronic and repeated stress, for example, results in increased release, turnover and synthesis of neurotransmitter serotonin and noradrenaline (Irwin et al., 1986; Adell et al., 1988; Nisenbaum et al., 1991), and enhances increased levels of expression of some genes, such as the stress-related

hormone, corticotropin-releasing factor (CRF: Herman et al., 1989; Imaki et al., 1991 and Mamalake et al., 1993).

Finally, the present results are important because work by others (using acute, hemorrhage treatment or chronic salt water drinking) indicates the degree of Fos expression in terms of immunoreactivity usually parallels the intensity of the applied stimulus (Roberts et al. 1993; Lafarga et al. 1993). I expected that Fos immunoreactivity would be increased or at least the same compared with what was seen after a single osmotic stimulus.

A good deal of further work, then, is needed to determine the significance of the present findings. Studies in the future should first determine the role of Fos and Jun in the transcriptional control of the two main hormones of the SON, vasopressin and oxytocin. Also, studies should be undertaken to outline what role other members of the Fos and Jun family play in this process. This work will then further allow us to understand molecular biological mechanisms that come to play in an important neuroendocrine system.

CHAPTER 3

Northern Blotting and *In Situ* Hybridization Analysis of c-*fos* and c-*jun* mRNA Expression in SON Neurons of Rats Following Acute and Repeated Fluid Imbalance

NORTHERN BLOTTING ANALYSIS OF c-*fos* AND c-*jun* mRNA EXPRESSION IN SON NEURONS OF RATS FOLLOWING ACUTE AND REPEATED FLUID IMBALANCE

Introduction

Northern blotting was used by Carter and Murphy (1990) to study the expression of c-*fos* and c-*jun* mRNAs in SON neurons derived from rats administered acute hypertonic saline. They found there was a 6-fold increase in SON c-*fos* mRNA at 1 hour and a 3.5-fold increase by 2 hours after saline injection. Changes in c-*jun* mRNA were less consistent, although increased levels were evident 1 hour after injection (Carter et al., 1990). Using the same technique, I first determined the time course of c-*fos* and c-*jun* mRNA expression. As my previous work has shown that c-Fos and c-Jun protein expression is less in SON tissue

from rats that received a repeated osmotic stimulus, I attempted to determine if mRNA levels for c-*fos* and c-*jun* gene were also attenuated by this experimental treatment.

Materials and Methods

Preparation of Hybridization Probes to c-*fos* and c-*jun* mRNA

Transfection of Plasmids. The plasmids containing the rat clone pSP65 c-*fos* (2.2 Kb) 1A (antisense), pSP65 c-*fos* (2.2Kb) 1B (sense) and pGEM-4 c-*jun* (1.8 Kb) were kindly provided by Dr. Curran (Department of Molecular Oncology and Virology, The Roche Institute of Molecular Biology, Nutley, NJ) (see Curran et al., 1987) . Frozen competent *E. coli* (DHα5) cells (American Type Culture Collection, Rockville, MD) were thawed in ice for 30 min. Aliquots (70 μl) of cells were transferred to a 1.5 ml tube containing 10 μl cDNA samples (5 ng/μl). The tubes were gently mixed, incubated on ice for 30 min, and then heat-shocked at 37°C for 2-5 min (or 42°C for 45 sec-2 min) before placement on ice for 2 min. LB medium (0.9 ml) was added to the tube before it was shaken vigorously at 37°C for 1 hr. Aliquots (up to 100 μl undiluted cells) of transformation culture were plated on LB/ampicillin antibiotic-containing plates. When the plates were dry, the plates were incubated for 12-16 hr at 37°C. The remainder of the transformation culture were stored at 4°C for subsequent plating.

Extraction and Purification of Plasmid DNA. A single bacterial colony was transferred into 10 ml of LB liquid medium supplemented with 500 μg of ampicillin (final concentration: 50 μg/ml of media) and incubated with vigorous shaking at 37°C for 18 hr. The cultures (1.5 ml of each) then were transferred to sterile 1.5 ml Eppendorf tubes and centrifuged at 12,000 x g for 2 min at 4°C. The supernatant were discarded and each pellet was suspended in 350 μl of freshly prepared STET buffer [8.0 % (w/v) sucrose, 5 % (w/v) Triton X-100, 50 mM disodium EDTA, 50 mM Tris (pH 8.0)], plus 25 μl of a freshly prepared solution of lysozyme [10 mg/ml in 10 mM Tris-HCl, pH 8.0]. These tubes were placed in a boiling water bath for 2 min and then centrifuged for 15 min at room temperature. The supernatant was transferred to new sterile 1.5 ml Eppendorf tubes and 320 μl of isopropanol was added to each supernatant. These tubes were left on ice for at least 5 min before centrifugation of the DNA in a Micro-Centaur for 15 min at 4°C. After discarding the alcohol, pellets were allowed to air-dry for 30-40 min. The nucleic acid pellets were dissolved in 50 μl of dH_2O, vortexed briefly, and stored at -20°C.

Digestion of DNA with Restriction Endonuclease. The purified plasmid DNA (100 μl: 3μg/μl) were placed in a sterile microfuge tube and diluted in water (final volume:

135 µl). RNase solution (9 µl) and 16 µl of the 10x restriction enzyme digestion buffer (Buffer H, Boehringer Mannheim, Indianapolis,IN) were added to the tube with 5 units of *EcoRI* restriction enzyme (Boehringer Mannheim, Indianapolis,IN). The mixture were incubated at 37°C for 1 hr. The reaction was stopped and prepared for agarose gel electrophoresis by adding 28 µl 6x DNA gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water).

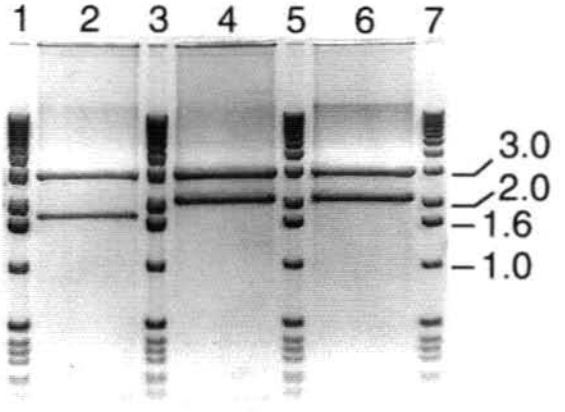
Gel Electrophoresis of DNA. An adequate volume of 1x TBE buffer was prepared to fill the electrophoresis tank to separate the nucleic acids on a 0.8% agarose gel. The gel (2.4 g agarose/300 ml 1x TBE buffer) was prepared by melting the gel in a microwave oven; 15µl ethidium bromide (10mg/ml) was added to the melted gel when it cooled to 55°C. After the gel had hardened, the digested DNA samples and a 1 Kb DNA Ladder (Gibco-BRL-Life Tech., Inc. Gaithersburg, MD) was loaded for electrophoresis (58 V constant voltage) at room temperature for 18 hr.

Purification of DNA Fragments. The gel was placed on a light box and the 2.2 Kb c-*fos* and the 1.8 Kb c-*jun* fragments were cut out with a razor blade (Fig. 9). The agarose fragments were centrifuged at 50 rpm (14°C) for 20 min in a Beckman TLA-100 centrifuge. The supernatant was

transferred to a 1.5 ml tube and extracted with a half volume of 0.1 M Tris pH 8.0-saturated phenol. After centrifugation, the aqueous phase was transferred to a new 1.5 ml tube, and an equal volume of chloroform was added. The tubes were vortexed, centrifuged, and extracted a second time using chloroform. The aqueous phase was removed and the DNA was ethanol precipitated (1/10 vol Na-acetate). The air-dried DNA was then dissolved in 10 μl water.

cDNA Labeling by Random Priming. A random primed DNA labeling kit (Pharmacia Biotech, Piscataway, NJ) was used for the following reaction. Sterile tubes containing either the antisense sequence of c-*fos* or antisense c-*jun* cDNAs (250-350 ng) were diluted in dH_2O (the total volume to 34 μl), heated for 10 min, and then cooled in ice-water. The following reagents were then added: 10 μl reaction buffer, 5 μl [^{32}P]-dCTP, and 1 μl Klenow enzyme. The reaction was mixed and incubated at 37°C for 1 hr. The reaction was stopped by adding 2 μl of 0.2 M EDTA (pH 8.0) and purified by passing the G-50 microcentrifuge spin columns (5 Prime→3 Prime, Inc. Boulder, CO). The reaction solution was monitored by removing 1μl samples for scintillation counting.

Figure 9. An 0.8% agarose gel was electrophoresed to separate plasmid DNA digested by an *EcoRI* restriction enzyme. Lanes 1, 3, 5, and 7 are a 1-Kb DNA ladder. Lane 2 is the plasmid containing a rat clone pGEM-4 c-*jun* (antisense). Lane 4 is the plasmid containing rat clone pSP65 c-*fos* cDNA (antisense). Lane 6 is the plasmid containing the rat clone pSP65 c-*fos* cDNA (sense).


1
2
3
4
5
6
7
3.0
2.0
1.6
1.0

Synthesis of a Probe to Actin by Single-Stranded PCR

To control for variations in the amount of RNA applied to each lane of the gel, an actin probe was prepared to assess the amount of this message in each RNA sample. A chicken cytoplasmic ß-actin template and 3' primer (3-prime-5'-GCTTGACCCTACTATACCTC-3') was kindly provided by Dr. Rita Dhawan (Dhawan, 1994). The amplification of ^{32}P-labeled cDNA probe was performed using a programmable thermal controller (MJ Research, Inc., Watertown, MA). The following reagents were mixed and overlaid with 20 μl of mineral oil: 1 μl of cDNA template (10ng/μl), 2 μl of 3' primer (767), 2 μl of Taq DNA polymerase reaction buffer with 1.5 mM of $MgCl_2$ (Promega, Madison, WI), 1.6 μl of dNTP (except of dCTP, 2.5mM each), 12.4 μl of ^{32}P dCTP (3000Ci/mmol), and 1 μl of Taq DNA polymerase (5000 Unit/ml, Promega, Madison, WI). PCR consisted of denaturation at 94 °C for 40 seconds, followed by annealing at 55 °C for 1 min and extension at 72 °C for 1 min. The single-stranded PCR reaction was amplified for 40 cycles, and the labeled probe was purified by passing the reaction solution through an Ultrafree-MC column (Millipore Corporation, Bedford, MA).

Animal Treatment for c-*fos* and c-*jun* mRNA Expression in SON Neurons of Rats Following Acute Fluid Imbalance

Twenty male Long-Evans rats were housed as described above. The animals were divided evenly (n=4 rats per group) and received an intraperitoneal injection of 1.5 M hypertonic saline. The animals were anesthetized and decapitated after the following survival times: 5, 15, 30-60, 120 and 180 min. The brains were removed, positioned in a custom mold, and immediately sliced into coronal sections (1 mm of thickness) with a razor blade. The slices were placed on a flat surface cooled with dry ice, and the tissue containing the SON was punched with a sterile N0. 16 blunt needle.

Animal Treatment for c-*fos* and c-*jun* mRNA Expression in SON Neurons of Rats Following *Repeated* Fluid Imbalance

Twenty male, Long-Evans rats were randomly assigned to receive one of following treatments; the procedure is summarized in the table below. Group 1 received a normal saline injection and were then returned to their cage for 3 hours before receiving a second saline injection. These rats were then sacrificed 30 minutes later. The rats of Group 2 first received a 0.9% saline injection, and then a

1.5 M hypertonic injection. Group 3 was used as positive control group, receiving a single 1.5 M hypertonic injection and a 30 minute survival time. Group 4 received the two 1.5 M hypertonic saline injections. Group 5 was used as a negative control group and received no injection. All rats were sacrificed using the procedure described earlier. Plasma osmolality and sodium content data were assessed for each group, using the procedure outlined previously. For reasons that remain unknown (we believe it may have been a machine malfunction), blood values for the rats in Group 5 were inordinately low and essentially not compatible with life (mean group osmolality and sodium levels, 201 and 101, respectively). These data were eliminated and were not included in the statistical analyses.

Northern Blotting

RNase-free conditions were employed during all procedures. The mini-filtration apparatus was rendered RNase-free by immersion in AbSolve (Du Pont, Wilmington, DE) for 30 min followed by rinses with diethylpyrocarbonate-treated (DEPC: Sigma Chemical Co.) water. Gloves were worn at all times, and pipettes, microfuge tubes, etc. were autoclaved prior to use.

Table 2

Summary of Injection Scheme and Time Course for Treatment Groups

Group (n=5)	1st Injection	2nd Injection	Survival Time
1	0.9% NaCl	0.9 % NaCl	3 hr + 30 min
2	0.9% NaCl	1.5 M NaCl	3 hr + 30 min
3	1.5 M NaCl	____	30 min
4	1.5 M NaCl	1.5 M NaCl	3 hr + 30 min
5	No Injection		

Isolation of total RNA. Total RNA were extracted from the SON using RNAzol™ B Isolation of RNA Kit (TEL-TEST,INC. Friendswood, TX). The punched tissue were pooled into a glass-Teflon homogenizer, and minced with 0.8ml of RNAzol™ B. The tissue homogenate then were transferred to 1.5 ml microcentrifuge tubes. Chloroform (80 μl) was added and the samples was stored for 5 min at 4°C. The phases were separated by centrifugation at 12,000 x g for 15 min. The supernatant (aqueous phase) was then transferred to a new tube, and 0.4 ml of isopropanol was added. The nucleic acids were allowed to precipitate at -4°C for 45 min. The RNA precipitates were centrifuged for 15 min and washed once with 0.8 ml of 75% ethanol. After centrifugation, the supernatant solution was carefully removed from the tubes. The pellets were dried in a vaculum desiccator for 15 min and resuspended in 50 μl of RNase-free dH_2O/0.1 mM EDTA with intermittent agitation on a vortex and incubation on a heat block (70°C) for 3 min. The RNA samples were stored in a -70°C freezer.

Electrophoresis. A 1.2% agarose gel was prepared by dissolving 0.24 g agarose in 16.5 ml dH_2O with 2ml of 10x MOPS buffer [0.2 M MOPS 3-(N-morpholino) propanesulfonic acid (Sigma, St. Louis, MO), 50mM sodium acetate, 10mM EDTA (pH = 7.0 by titration with NaOH)] at 100°C. The melted agarose was cooled to 65°C, and 1.75 ml of 37% formaldehyde

(Mallinckrodt) was added.

Sample Preparation. Up to 20 μg of total RNA was loaded per lane. Each sample was prepared as follows: 10 μl of loading buffer [0.72 ml formamide, 0.16 ml 10 X MOPS buffer, 0.26 ml formaldehyde(37%), 0.18 ml DEPC-H_2O, 0.1ml 80% glycerol, and 0.08 ml bromophenol blue (saturated solution)] were added to each sample, the tubes spun, incubated at 65°C for 12-15 min, and quenched on ice. RNA molecular weight markers was also prepared [Bethesda Research Laboratories (BRL), Gaithersburg, MD] as described previously. The gel was run at 82 mV for 1 hr or until the bromophenol blue dye migrated three-fourths of the distance down the gel. Using a ruler as a scale marker, the gel was photographed on a UV light gel box.

Blotting Transfer of RNA. The gel was rinsed for 20 min each in two changes (200 ml) of 10x SSC to remove formaldehyde from the gel. The tray was filled with 500 ml of 10x SSC, a glass plate was placed over the tray and a wet piece of Whatman 3MM filter was wrapped over the plate with both ends hanging into the buffer to act as a wick. Any bubbles or lumps in the filter were smoothed out with a glass rod. The gel were placed upside down over the wick. A nylon membrane filter (Nytran, Schleicher and Schuell), wetted in DEPC-H_2O, was placed on the top of the gel and

smoothed. Two pieces of Whatman 3MM paper and paper towels were then laid flat and smooth on the top of the Nytran filter to initiate transfer. A glass plate covered the paper towels with a small weight on top for 12-24 hr at room temperature. The next day, the Nytran filters, placed on a sheet of Whatman 3MM filter, first crosslinked using the Stratalinker UV crosslinker (Stratagene, La Jolla, CA), and then baked for 2 hr under vacuum at 80°C.

Prehybridization. The filter containing the immobilized RNA was incubated in prehybridization buffer (4 ml) at 65°C for 2 hr. [Prehybridization buffer: 50% formamide, 6x SSPE [20x SSPE: 3.6 M NaCl; 0.2 M sodium phosphate, pH 7.7; 1mM EDTA], 5x Denhardt's [50x Denhardt's reagent: 1.0% (w/v) each of ficoll, polyvinylpyrrolidone, and bovine serum albumin], 0.5% (w/v) sodium dodecyl sulfate (SDS), and 100 μg/ml denatured salmon sperm DNA.]

Hybridization. The ^{32}P labeled cDNA c-*fos* and c-*jun* probes were prepared as outlined previously. The probes were mixed with salmon sperm DNA (100 μg/ml HB), heated for 10 min in boiled water, and then quickly immersed in ice water. The probe ($1x10^6$ cpm/ml HB) was mixed with hybridization buffer and added to the filter to hybridize overnight at 45°C. [The hybridization buffer: 50% formamide, 6X SSPE, 0.5% SDS.]

Posthybridization Treatment. The filter was immersed in 2x SDS, then in 0.2% SDS at room temperature for a few min, followed by washing twice for 20 min in 2x SSPE, 0.1% SDS (45°C). The filter was then wrapped in plastic membrane and placed in a cassette against Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) with intensifying screens (Sigma, Louis, MO). Films were exposed overnight or up to 6 days (-70°C).

Analysis and Quantitation of Northern Results. Densitometric analysis of the autoradiographic films from the Northern hybridization blots was performed using the NIH Image 1.4 image analysis software (Developed by Wayne Rasband, NIH, Bethesda, MD). Total RNA concentration dilutions of 4, 8, 12, 16, 20 μg from SON neurons of rats that had received hypertonic saline injections were used to generate a densitometric standard curve for determining relative c-*fos* and c-*jun* mRNA content.

Results

c-*fos* and c-*jun* mRNA Expression in SON Neurons of Rats Following Acute Fluid Imbalance

Northern blot analysis (Figure 10) identified single bands of mRNA for c-*fos* and c-*jun* of 2.2 Kb and 2.7 Kb, respectively. Pixel density measures of the northern blots after hybridization with the two probes is summarized in Figure 11. Increasing amounts of total RNA from the SON tissue punches resulted in increasingly higher pixel density measures.

Figure 12 shows results from hybridization of tissues from rats that were sacrificed at different time points after hypertonic saline administration. For the time course study, separate blots were prepared for hybridization to c-*fos* and c-*jun* probes, and in each case a single band hybridized to each probe. Each blot was then stripped and re-hybridized with an actin probe. The induction of both c-*fos* and c-*jun* mRNAs occurred within 5 minutes, peaked at 30 to 60 minutes and gradually disappeared by 3 hours. Compared to levels seen in rats that were sacrificed with 5 minutes of saline administration, there was a 4-fold increase in SON c-*fos* mRNA at 30 min and 2-fold increase at

2 hr, there was a 2.6-fold increase c-*jun* mRNA in 30 min and 1.7 fold increase at 2 hr (Figure 13). Hypertonic saline injection appeared to increase c-*fos* mRNA levels to a greater extent than c-*jun* mRNA levels.

Figure 10. Northern blot experiment using increasing amounts of total RNA from SON tissue punches taken from 5 rats that had all received an injection of hypertonic saline. Blots were probed and stripped sequentially with ^{32}P-labeled probes specific for c-*fos*, c-*jun* and actin mRNA. The ß-actin cDNA was used to control for differences in the amount of RNA applied to each lane of the gel.

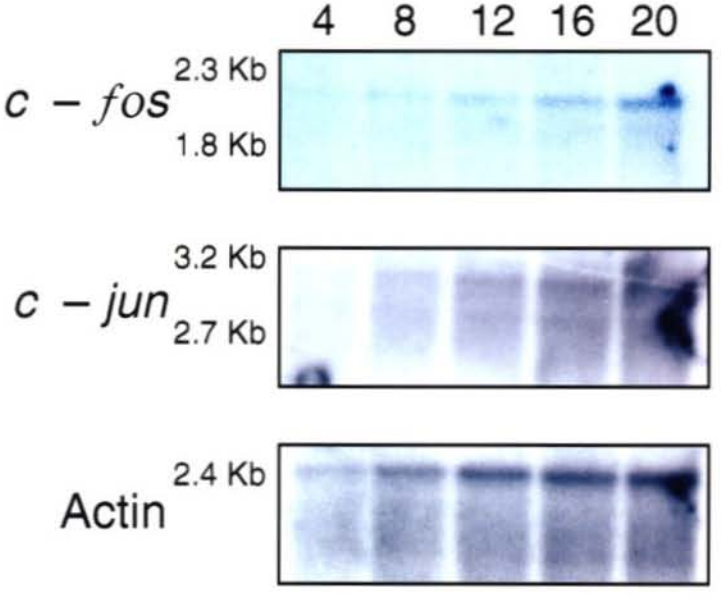
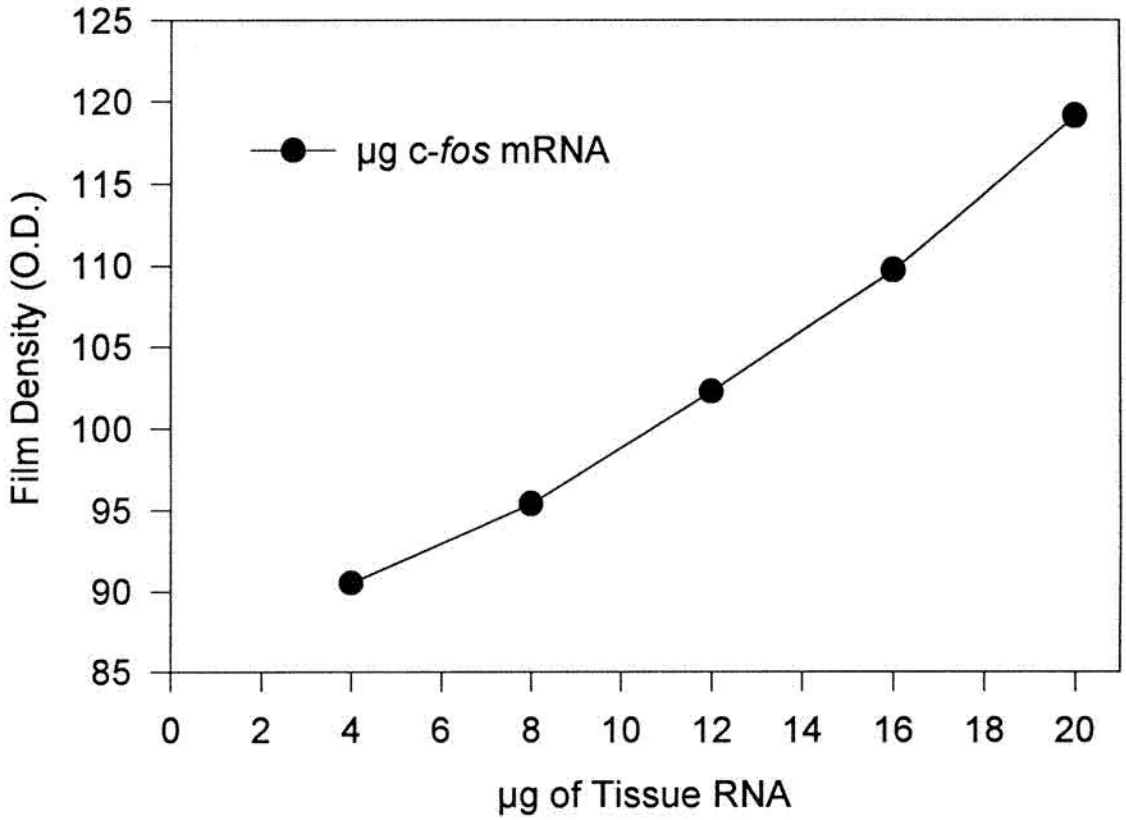

4
8
12
16
20
c – fos
2.3 Kb
1.8 Kb
c – jun
3.2 Kb
2.7 Kb
Actin
2.4 Kb

Figure 11. Pixel density of film autoradiograms from northern hybridization as a function of μg of tissue total RNA extracted from pooled SONs (n=5). RNA was obtained from rats that had received a hypertonic saline injection 30 minutes prior to sacrifice.

Optical Density of Film Autoradiograms *vs.* µg of Tissue Total RNA



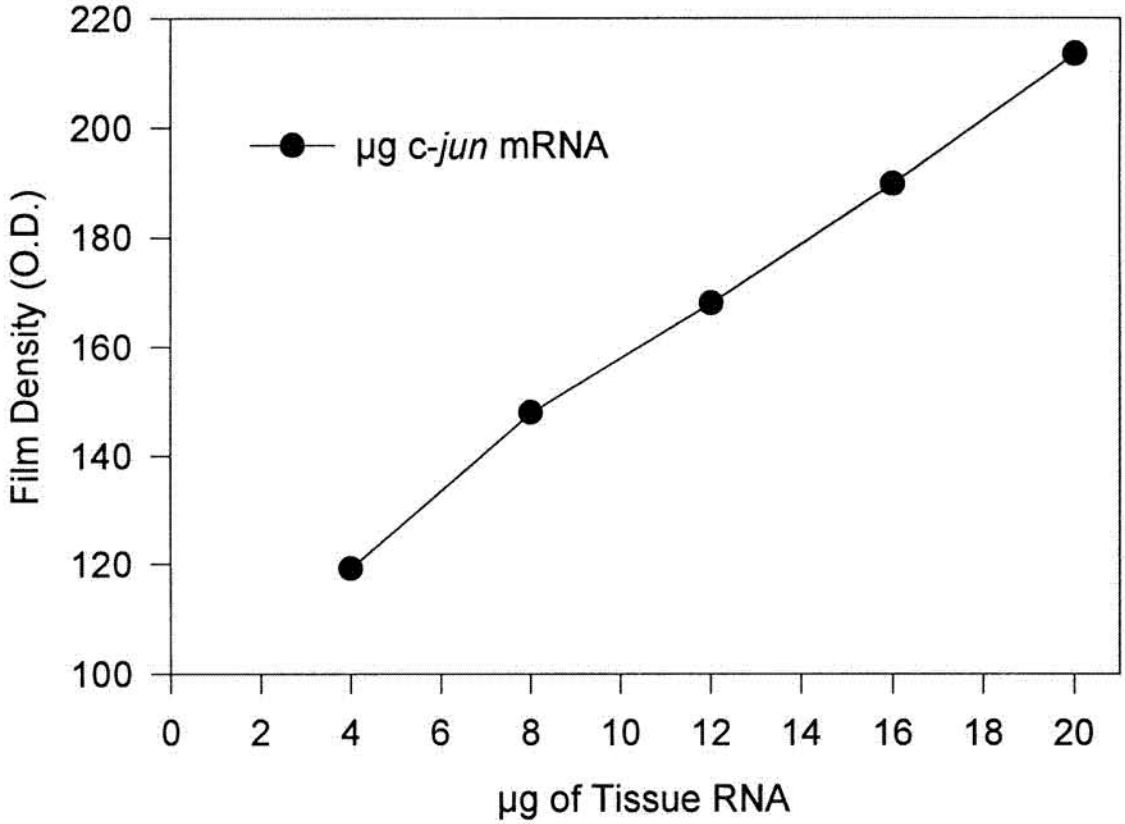


Figure 12. Northern blots of total RNA (20 μg/lane) extracted from pooled SON (n=4) taken from male Long-Even rats 5, 15, 30, 120, and 180 min after a hypertonic saline injection. Blots were probed and stripped sequentially with ^{32}P-labeled probes specific for c-*fos* and actin mRNA, or for c-*jun* and actin mRNA. The c-*fos* blot is a one-day exposure; the c-*jun* blot, a five-day exposure. The ß-actin cDNA was used to control for differences in the amount of RNA applied to each lane of the gel.

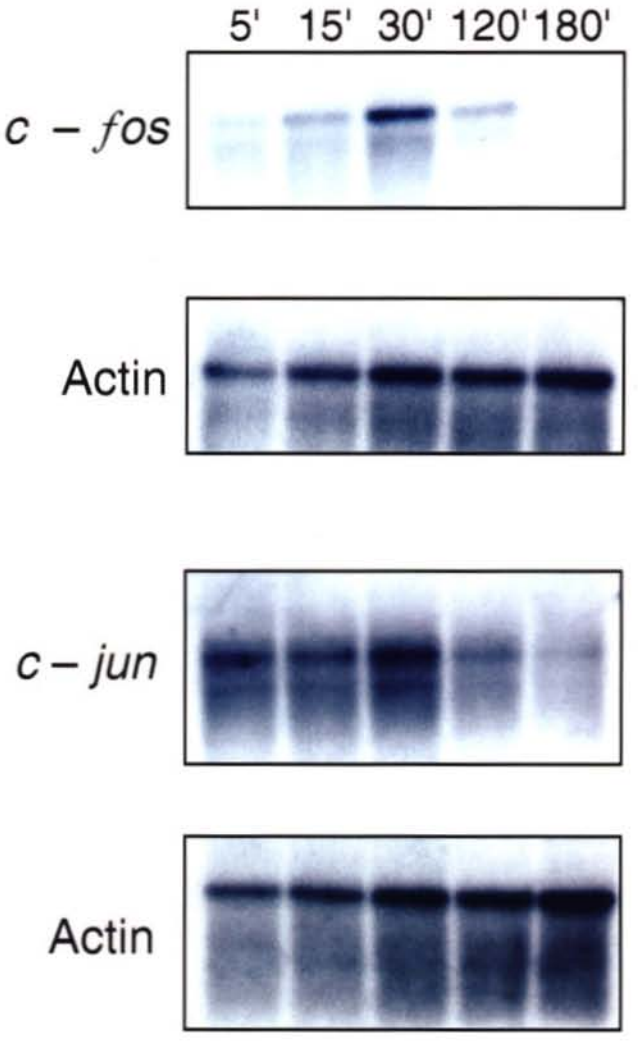
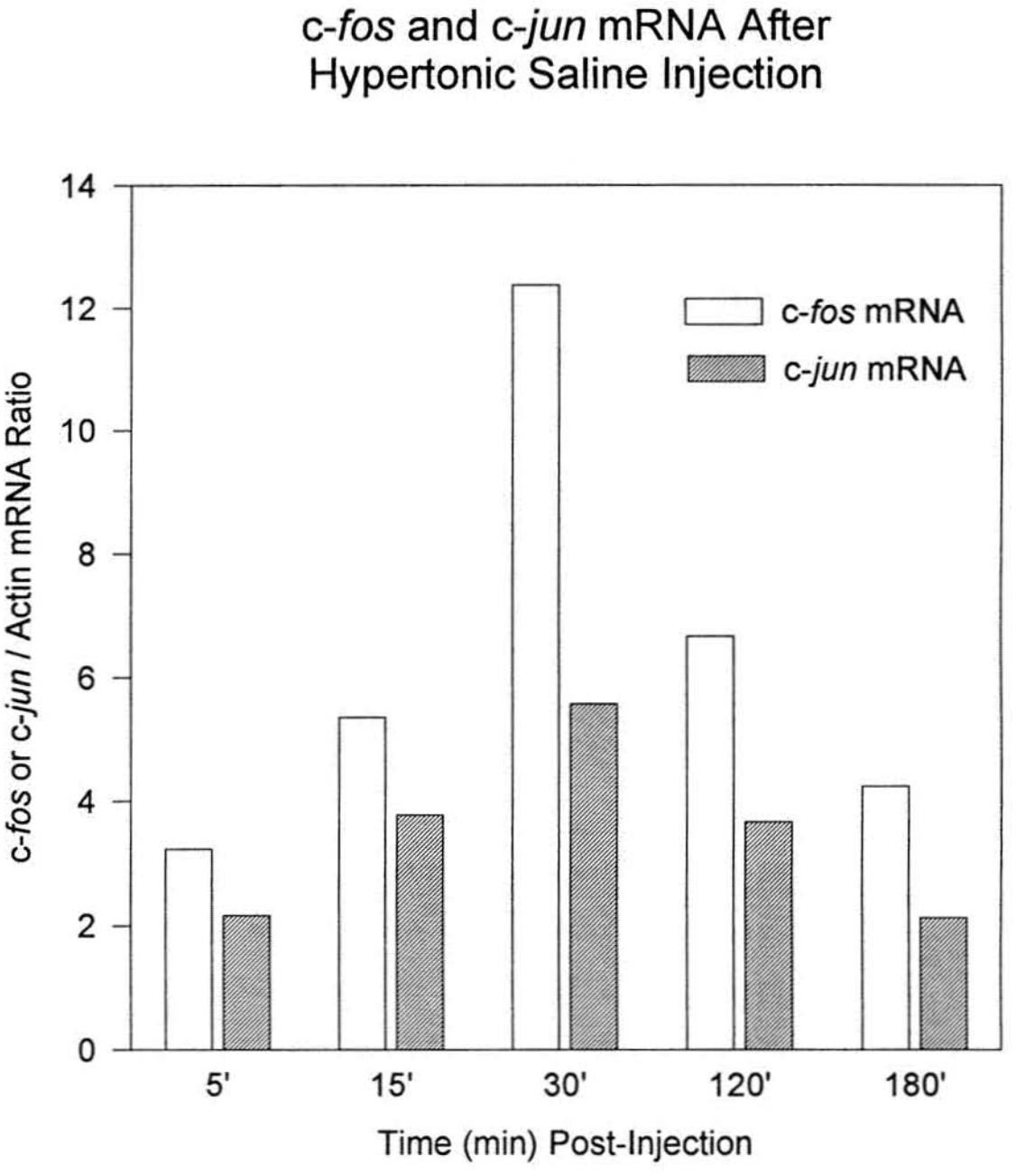

5'
15'
30'
120'
180'
c – fos
Actin
c – jun
Actin

Figure 13. Rats were sacrificed at selected time points after a single injection of hypertonic saline. Changes in c-*fos* and c-*jun* mRNA levels were assessed relative to hybridization to an actin probe, the latter not believed to be significantly altered by saline administration.


c-fos and c-jun mRNA After
Hypertonic Saline Injection
c-fos mRNA
c-jun mRNA
c-fos or c-jun / Actin mRNA Ratio
0
2
4
6
8
10
12
14
5'
15'
30'
120'
180'
Time (min) Post-Injection

c-*fos* and c-*jun* mRNA Expression in SON Neurons of Rats Following *Repeated* Fluid Imbalance

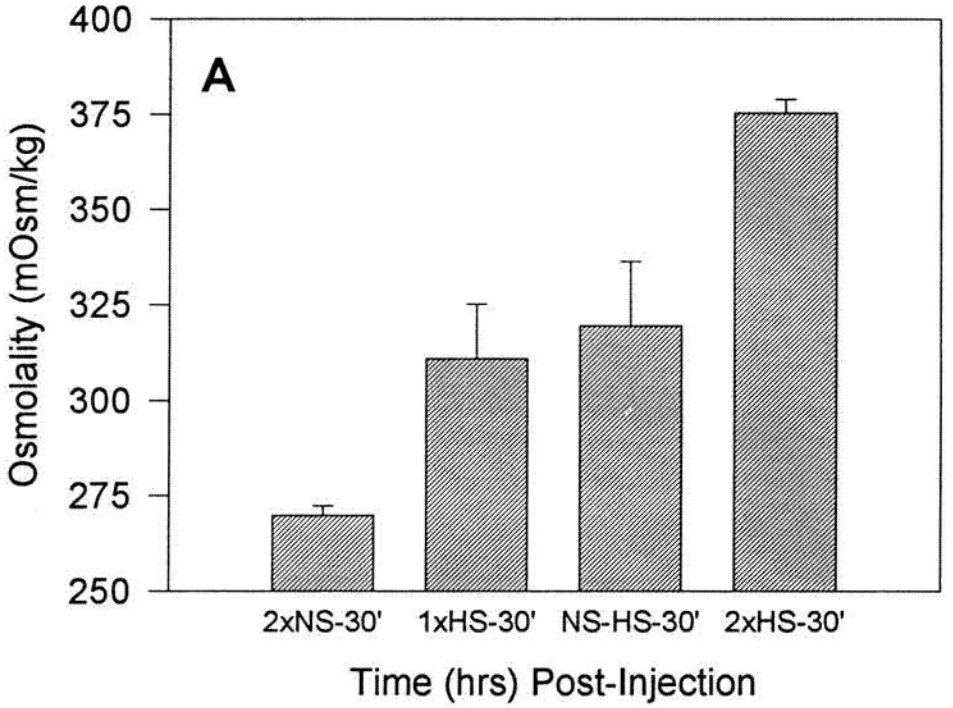
Compared with rats that received two normal saline injections (**2XNS-30'**), rats that were administered either one (**1XHS-30' or NS-HS-30'**) or two (**2XHS-30'**) intraperitoneal injections of hypertonic saline exhibited higher values for plasma osmolality and sodium concentration (Figures 14A and B, respectively). The rats that received two hypertonic saline injections (**2XHS-30'**) had significantly higher osmolality and sodium levels than all other groups (Bonferroni's test, $p < 0.05$). There was no difference in osmolality or sodium values for the rats that had received just one hypertonic saline injection whether or not it was preceded by a normal saline injection (**1XHS-30' or NS-HS-30'**).

Results from Northern hybridization (Figure 15) indicated that rats which were administered either one (**1XHS-30' or NS-HS-30'**) or two (**2XHS-30'**) intraperitoneal hypertonic saline injections exhibited an increase in c-*fos* mRNA expression compared with groups of animals that received no injection (**Co**) or two normal saline injections (**2XNS-30'**). All groups of animals exhibited c-*jun* mRNA expression. Compared with non-injected and two normal saline injection (**2XNS-30'**) rats, there was no difference

for c-*jun* mRNA expression in two hypertonic saline injected rats (**2XHS-30'**). However, the expression of both c-*fos* and c-*jun* mRNA in rats that received two hypertonic saline injections (**2XHS-30'**) was less compared with rats that received just one hypertonic saline injection (**1XHS-30' or NS-HS-30'**). The latter groups, however, did not differ.

Figure 14. Plasma osmolality and sodium concentration. **2XNS-30':** Rats received a normal saline injection and were then returned to their home cage for 3hr before receiving the second saline injection. These rats were then sacrificed 30 min later. **1XHS-30'** Rats was used as positive control group, receiving a 1.5 M hypertonic injection and a 30 min survival time. **NS-HS-30'** Rats first received 0.9% saline, and then a 1.5 M hypertonic injection. **2XHS-30'** Rats received the two 1.5 M hypertonic saline injections.

Plasma Osmolality (A) and Sodium Concentration (B) After Saline Injection Treatment



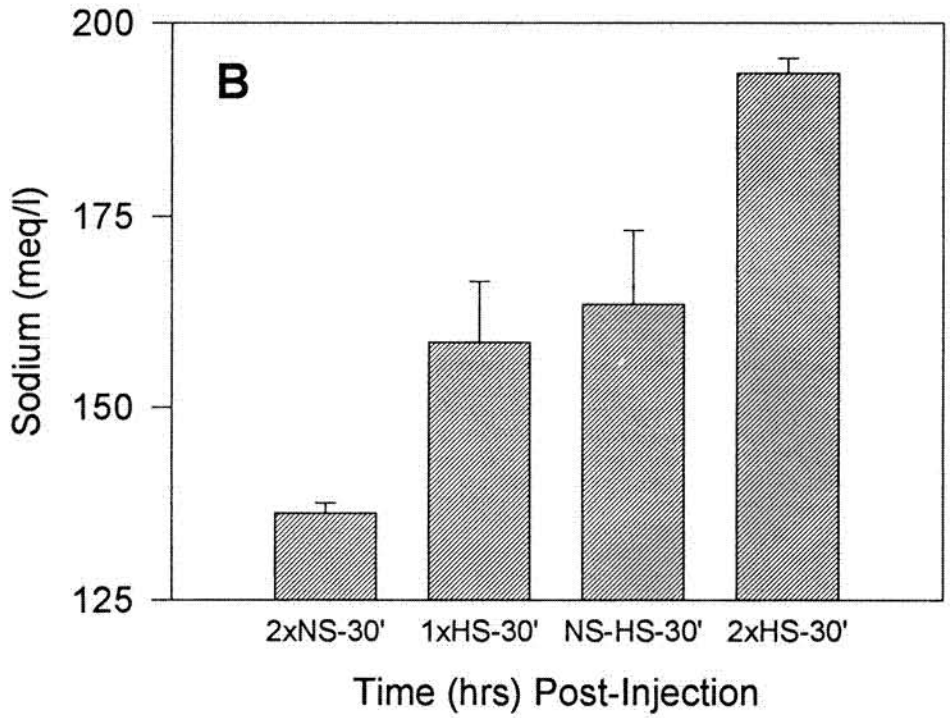
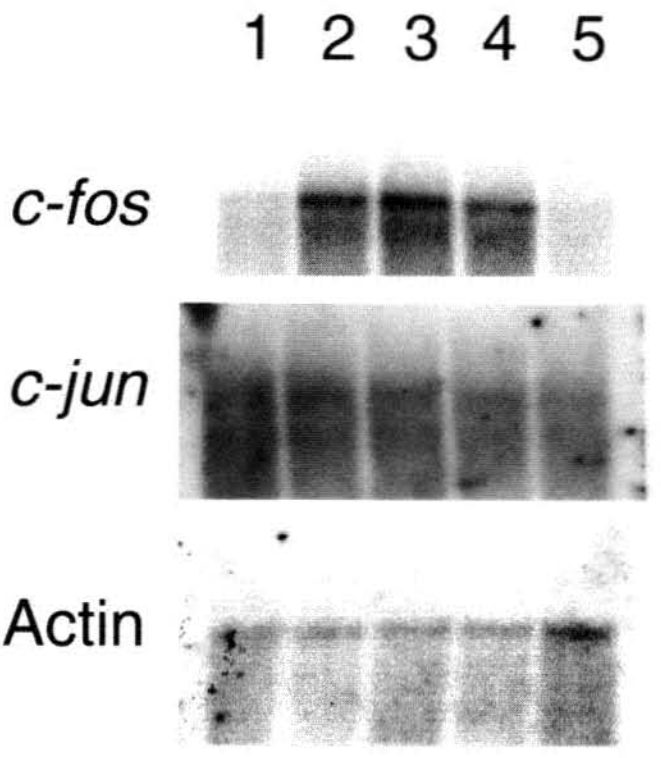


Figure 15. Northern analysis of total RNA (20 μg/lane) extracted from pooled SONs (n=4) taken from rats following various saline injection treatments. Lane **1**. Rats received a normal saline injection, were returned to their home cage for 3 hours, received a second saline injection, and then were sacrificed 30 minutes later (**2XNS-30'**). Lane **2**. Rats was used as a positive control group: they received a 1.5 M hypertonic injection and were sacrificed 30 minutes later (**1XHS-30'**). Line **3**. Rats first received 0.9% saline, and then a 1.5 M hypertonic injection (**NS-HS-30'**). Line **4**. Rats received the two 1.5 M hypertonic saline injections (**2XHS-30'**). Line **5**. Rats was used as a negative control group and received no injection (**Co**). Blots were probed and stripped sequentially with ^{32}P-labeled probes specific for c-*fos*, c-*jun*, and actin mRNA.


1 2 3 4 5
c-fos
c-jun
Actin

IN SITU HYBRIDIZATION ANALYSIS OF c-*fos* AND c-*jun* mRNA EXPRESSION IN SON NEURONS OF RATS FOLLOWING ACUTE AND REPEATED FLUID IMBALANCE

Introduction

Northern blot analysis was used to determine temporal changes in c-*fos* and c-*jun* mRNA levels acute hypertonic saline injection. This procedure employed punched tissue samples that included the SON, as well as the regions surrounding this nucleus. In the next experiment, I used *in situ* hybridization to more accurately determine the anatomical distribution of c-*fos* and c-*jun* mRNA. Similar to my previous work, I determined changes in mRNA levels as a function of time after acute saline injection. I then determined the effects of repeated hypertonic saline injection upon *fos* and c-*jun* mRNA levels in the SON.

Materials and Methods

Animal Treatments

Six groups of male Long-Evans rats (three per group) were housed as described earlier. On the day they were sacrificed, the animals received hypertonic saline intraperitoneally (1.5M/L NaCl) and then were allowed to survive for 0, 15, 30, 60, 120 or 180 minutes after injection. To test how repeated saline administration affects mRNA levels, three other groups of rats were randomly assigned to receive one of following treatments. Group 1 received a 1.5 M injection of saline, were returned to their cage for 3 hours, and then received a second hypertonic saline injection. These rats were then sacrificed 30 min later. Group 2 received similar treatment, except the two injections were 0.9% saline. Group 3 first received a 0.9% saline injection, and then a 1.5 M hypertonic saline injection. All rats were sacrificed using the procedure described earlier.

Tissue Preparation

Frozen brain blocks (-22°C) were cut in the coronal plane (12 μm sections) on a cryostat (Bright Cryostat,

Hacker Instruments, Fairfield, NJ) and mounted directly from the knife onto chrome-alum subbed microscope slides. Representative sections of the diencephalon were fixed in 4% paraformaldehyde, 0.1 M PBS with 0.2% diethylpyrocarbonate (DEPC) for 5 min, rinsed twice in 0.1 M PBS. Subsequently, the sections were placed in 0.25% acetic anhydride with 0.1 M triethanolamine HCl (pH 8) for 10 min, then were dehydrated in 70% ethanol, 80% ethanol, 95% ethanol, and 100% ethanol (1 min each), and then into a solution of 100% chloroform for 5 min, 100% ethanol and 95% ethanol for 1 min each. The slides were then stored in a vacuum desiccator.

Prehybridization

The slides were placed flat in air-tight boxes. The boxes were lined with a piece of filter paper, which had been saturated with Box buffer (4X SSC, 50% formamide). Each section was covered with 15 μl of hybridization buffer [HB: 50% deionized formamide, 10% dextran sulphate, 0.3M sodium chloride, 10mM Tris (pH 8.0), 1mM EDTA, 1X Denhardt's solution, 10mM DTT], and incubated at 43°C for 2 hrs. After incubation, the prehybridization solution was removed by gentle aspiration using a Pasteur pipette.

In Situ Hybridization

Microfuge tubes containing labeled c-*fos* or c-*jun* cDNA probes were mixed with salmon sperm DNA (100 μg/ml HB), heated for 10 min in boiling water, and then quickly immersed in ice water. The probe ($1x10^7$ cpm/ml HB) was further diluted by adding yeast t-RNA to the hybridization buffer and then each section was covered with 15 μl of the HB mixture for overnight incubation at 43°C.

Posthybridization Treatments

The slides were placed in metal slide racks and washed in 2xSSC, 1XSSC, 0.2XSSC, and 0.1XSSC for 1 hr each at 43°C with agitation on a shaker bath. The slides were rinsed for 1 min each in the following solutions of 300 mM ammonium acetate (pH 5.5): absolute ethanol at a 1:1 dilution (v/v: i.e., 400 ml ammonium acetate plus 400 ml ethanol), 3:7 (v/v), and 1:9 (v/v) solutions, and then in absolute ethanol, before they were air-dried in a desiccator under vacuum. The slides were placed against Hyperfilm-ß-Max (Amersham Corporation, Arlington Heights, IL) in a cassette with intensifying screens, and exposured for 2 days at -70°C. Quantitative densitometry of film images was accomplished by analyses with NIH IMAGE (version 1.49, Wayne Rasband, NIH, Bethesda, MD, see below).

Preparation of Autoradiograms

After exposure to film, the sections were prepared for emulsion autoradiography. A bottle of emulsion (NTB-3, Kodak) was allowed to come to room temperature over a 2-hr period, and then placed into a 43°C water bath. When the emulsion was warmed to 43°C, the slides were dipped and then air-dried for 1 hr in the darkroom, before they were placed in light-tight slide boxes that contain desiccant (United Desiccants-Gates, Pennsauken, NJ). The slide boxes were sealed with black vinyl electrical tape and stored at 4°C. Exposures ranged from 10 days to 3 weeks for the ^{32}P-labeled probes.

Development of the Autoradiograms

The slide boxes containing the *in situ* slides were removed from storage and allowed to warm up to room temperature. Under safelight conditions, the slides were loaded into slide racks and gently submerged in Kodak D-19 (16°C) for 2 min without agitation, then were dipped (10 sec) into water (19°C), and placed in Kodak Fixer (19°) for 10 min, with slight agitation after the initial 4 min of fixation. Slides then were rinsed in distilled water twice for 10 min each. The developed slides were then stained in a 0.5% cresyl violet solution for 10 min, washed in water,

and dehydrated in 70%, 95%, and 100% ethanol (one min each). The slides were cleared in Hemo-De for 1 hr and coverslipped with DPX or Permount.

Quantitation of *In Situ* Hybridization Results

Quantitative *in situ* hybridization was used to measure the level of c-*fos* and c-*jun* mRNA expression. Since there can be variation in tissue preparation and hybridization conditions across experiments (McCabe, et al., 1993), there can be difficulties in making meaningful comparisons across different hybridization trials. Therefore, all experimental (either one time or repeated hypertonic saline injected) and control animals were processed at same time with the same amount of radio isotopes. Tissue sections were carefully matched in order to compare as closely as possible identical fields in the brain.

X-ray film autoradiography was used for quantitative measurement by computerized image analyses densitometry. Although the density of the autoradiogram can not determine the exact copy of mRNAs hybridized, it permits the measurement of the percentage change of mRNAs expression level compared to the control sections. For each group of experimental and control animals, brain coronal sections containing SON neurons were analyzed. X-ray film

autoradiograms of brain sections were videoscanned and digitized. The hybridization signal areas were clearly visible and delimited by a cursor outline encompassing the magnocellular nuclei. A frequency histogram was used to select a threshold cutoff for hybridization signal from background averaged over the remaining areas of the brain section. Substraction of the outlined signal intensity by the tissue background yielded the normalized intensity. Statistical significance was determined using analysis of variance.

Results

In situ hybridization indicated that the induction of c-*fos* and c-*jun* mRNA expression was mainly in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) (Figure 16 and 18). Visual inspection of the films and autoradiograms (the emulsion-coated slides), indicated that levels of grain density were seen in the SON samples from rats that were sacrificed 30 min after injection of hypertonic saline. Signal was also detected in the subfornical organ (SFO, Figure 18), median preoptic nuclei (MnPO), and the lamina terminalis (LMT), but levels were never as intense as those observed in SON and PVN neurons. Hybridization signal was also observed in the nucleus circularis, the bed nucleus of the stria terminalis, periventricular regions of the hypothalamus, and the anterior nucleus of the hypothalamus (data not shown).

After hypertonic saline treatment, the expression of c-*fos* and c-*jun* mRNA in the SON of hypertonic treated rats was evident within 15 minutes, the signal intensity peaked by 30 to 60 minutes, and had disappeared by 3 hours after injection (see Figures 19 and 20). The increase in c-*fos* mRNA levels was much stronger and lasted longer than what was observed for c-*jun* mRNA. In control groups (non-

injected rats), there was no substantial increase in grain density to suggest high levels of c-*fos* or c-*jun* mRNA (data not shown) .

Compared with levels observed in SON tissue samples from rats that received one hypertonic saline injection (**1XHS-30'** or **NS-HS-30'**), the levels of c-*fos* and c-*jun* mRNA were significantly less in rats that had received two saline injections (**2XHS-30'**). Compared to rats that received no injections, rats that sustained two normal saline injections (**2XNS-30'**) were not different. The inhibition of c-*fos* mRNA expression after repeated hypertonic saline injection appeared to be less than that of c-*jun* (compare Figures 21 and 22).

Figure 16. c-*fos* (**A**,**B**) and c-*jun* (**C**,**D**) mRNA *in situ* hybridization coronal sections of brain from Long-Evans rats 30 min after hypertonic saline injection. **A** and **C**, dark-field; **B** and **D**, bright-field photomicrographs. *SON*, supraoptic nucleus; *PVN*, paraventricular nucleus; *3V*, third ventricle; *OC*, optic chiasm.

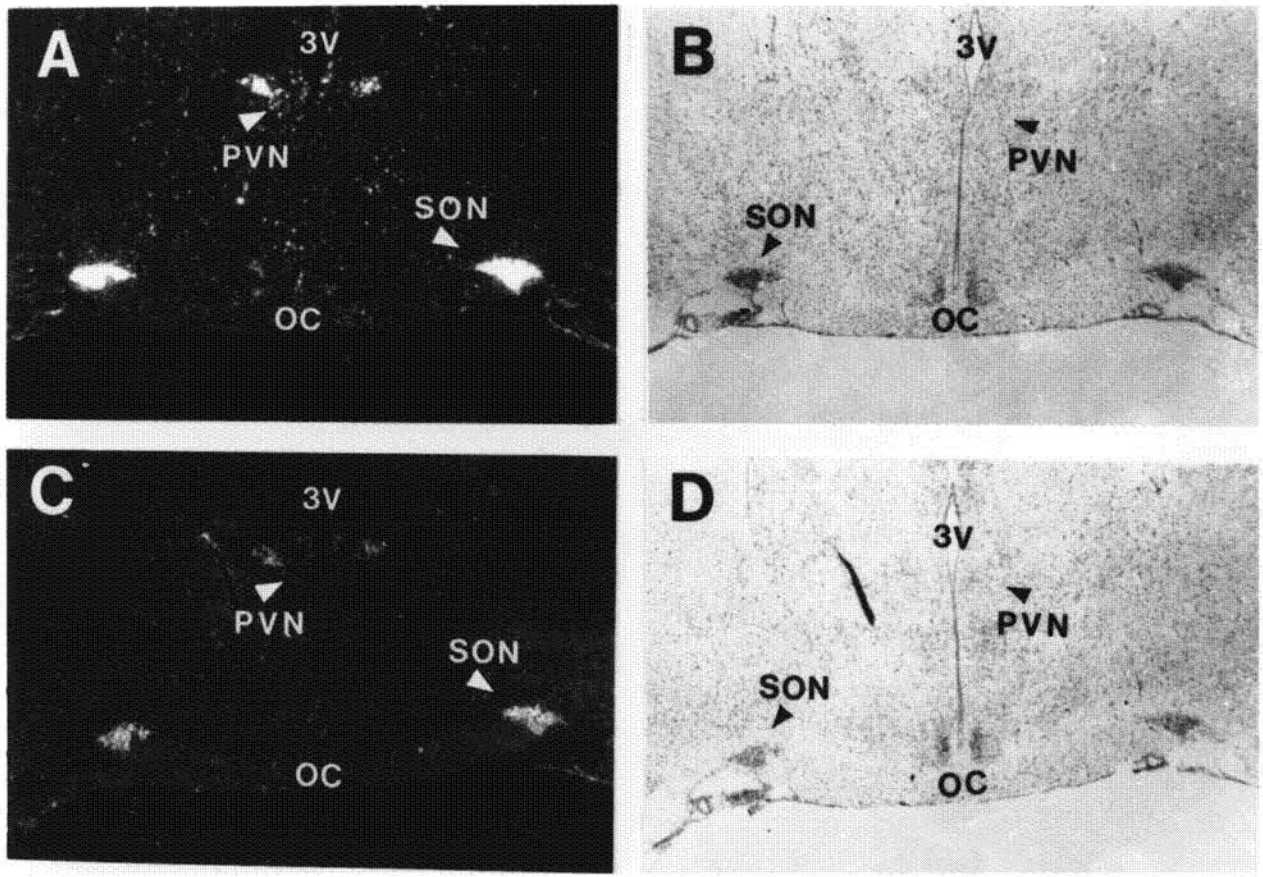

A
3V
PVN
SON
OC
B
3V
PVN
SON
OC
C
3V
PVN
SON
OC
D
3V
PVN
SON
OC

Figure 17. *in situ* hybridization shows c-*fos* (**A**) and c-*jun* (**B**) mRNA expression in magnocellular neurons in SON of Long-Evans rats 30 min after hypertonic saline injection (bright-field, high power).

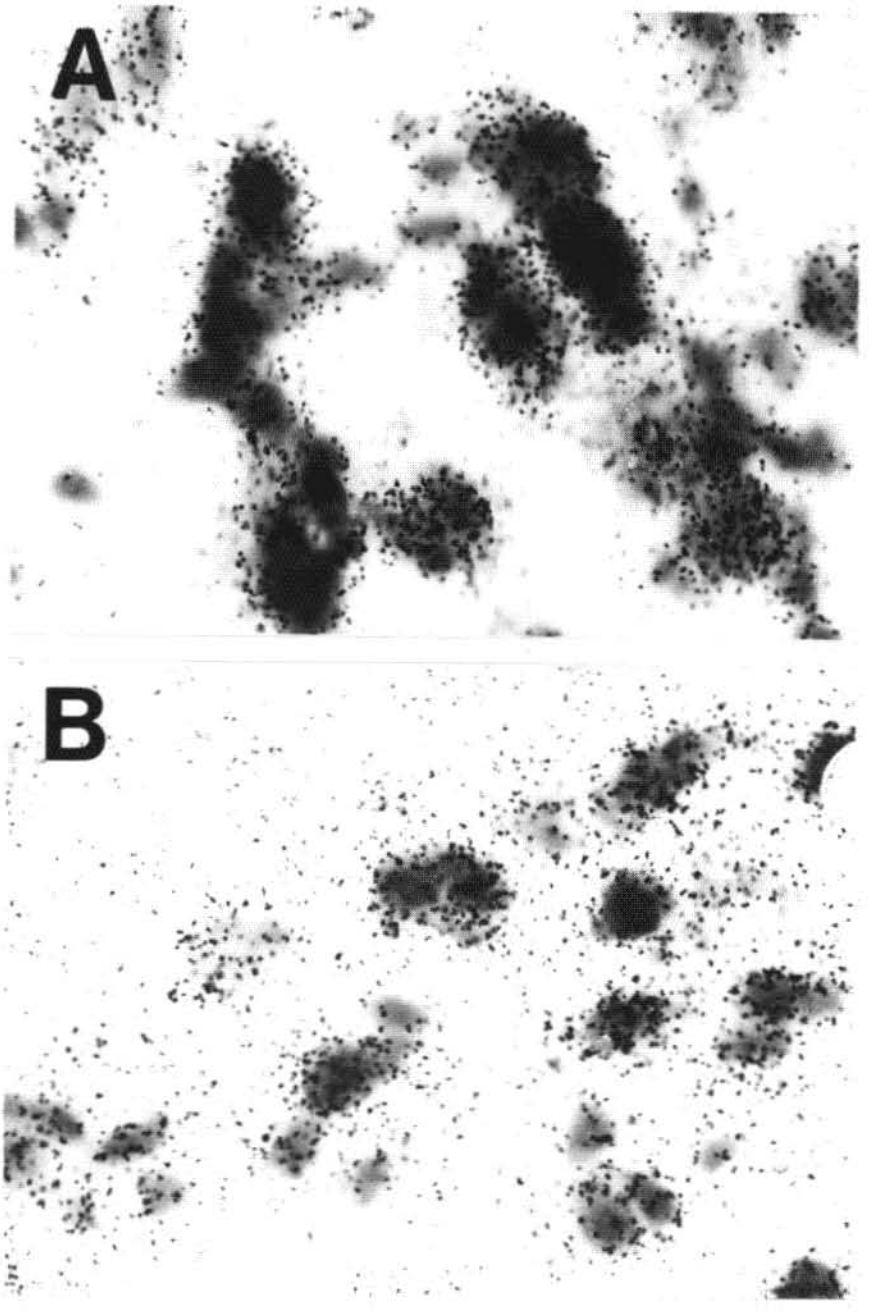

A
B

Figure 18. c-*fos* and c-*jun* mRNA *in situ* hybridization coronal sections 30 min after hypertonic saline injection. (dark-field photomicrographs) *SFO*, subfornical organ; *SON*, supraoptic nucleus; *PVN*, paraventricular nucleus; *3V*, third ventricle; *OC*, optic chiasm.

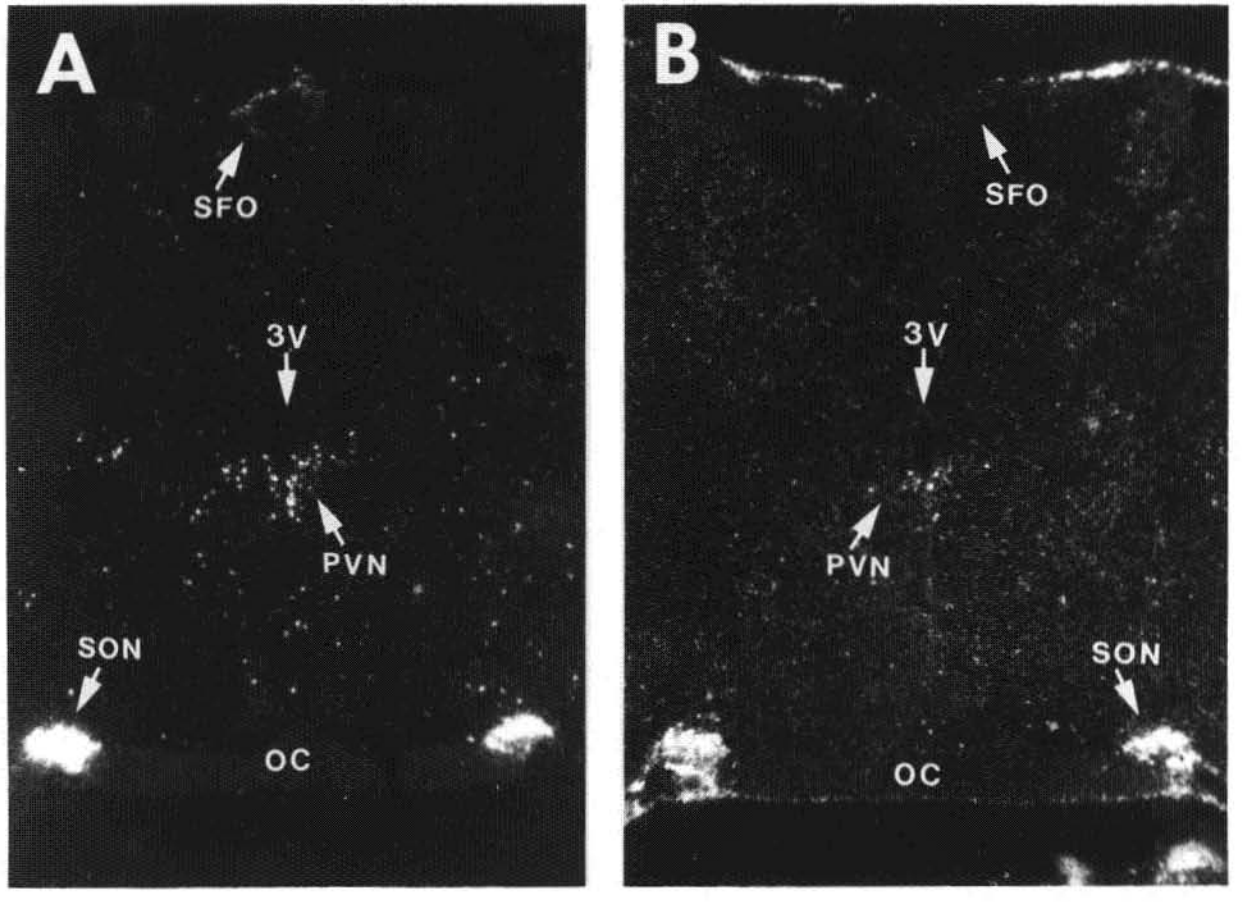

A
SFO
3V
PVN
SON
OC
B
SFO
3V
PVN
SON
OC

Figure 19. c-*fos* mRNA levels are shown after *in situ* hybridization of coronal brain sections through the region of the SON from rats, 0, 15, 30, 60, 120, and 180 minutes after receiving a hypertonic saline injection. The brain images were generated from computer capture of film autoradiograms, and color transformation has been used to increase visual perception of changes in pixel grey values. Red color indicates the highest degree of density of signal in the autoradiograms.

c-*fos* mRNA Expression in Hypothalamus after Acute Hypertonic Saline Injection

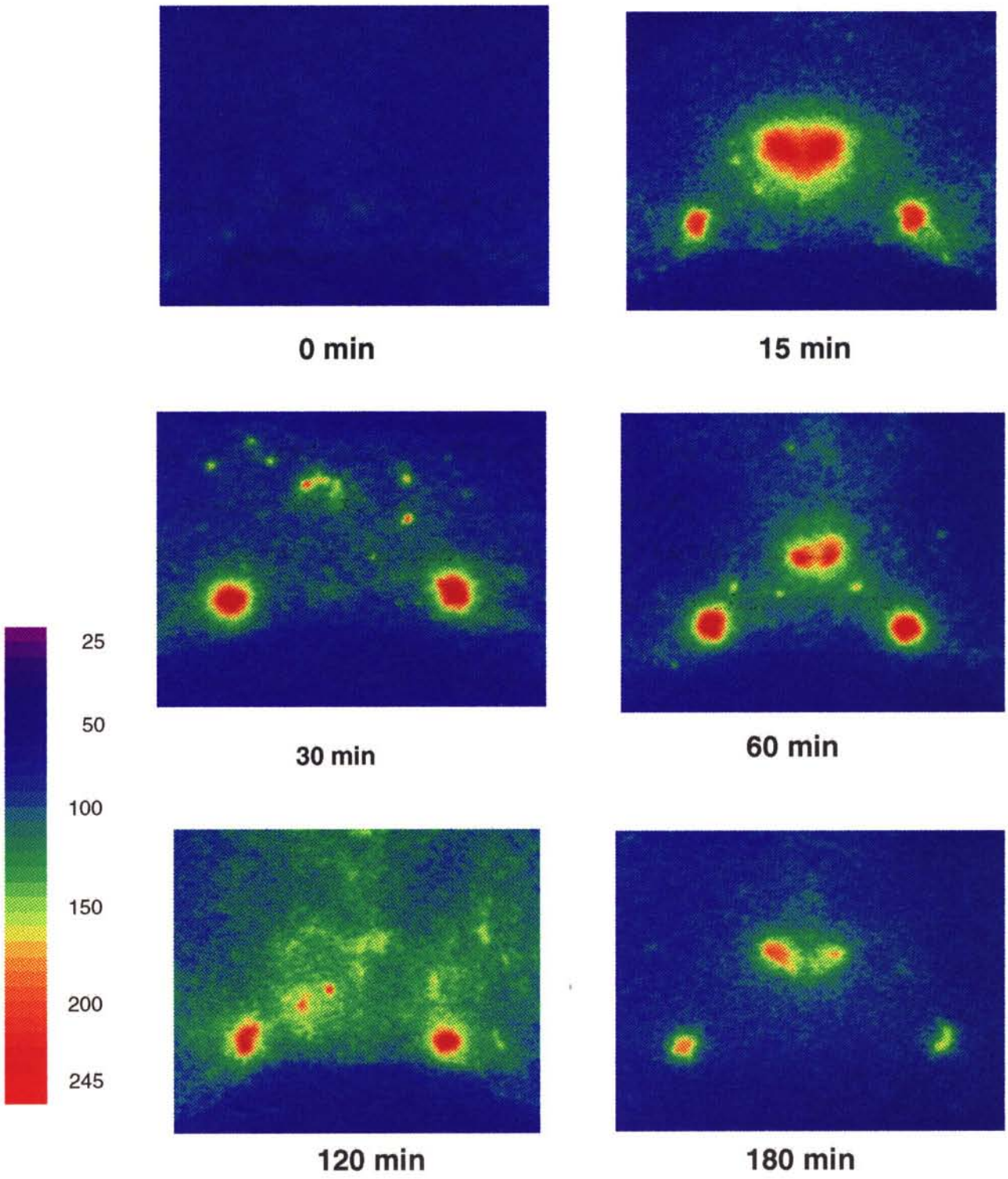


Figure 20. c-*jun* mRNA *in situ* hybridization results: coronal plane autoradiograms through brain sections containing the SON, from rats sacrificed 0, 15, 30, 60, 120, and 180 minutes after the administration of hypertonic saline. Increased levels of c-*jun* mRNA in the SON (and PVN) were evident within 15 min of injection, with levels peaking by 30 to 60 min.

c-*jun* mRNA Expression in Hypothalamus after Acute Hypertonic Saline Injection

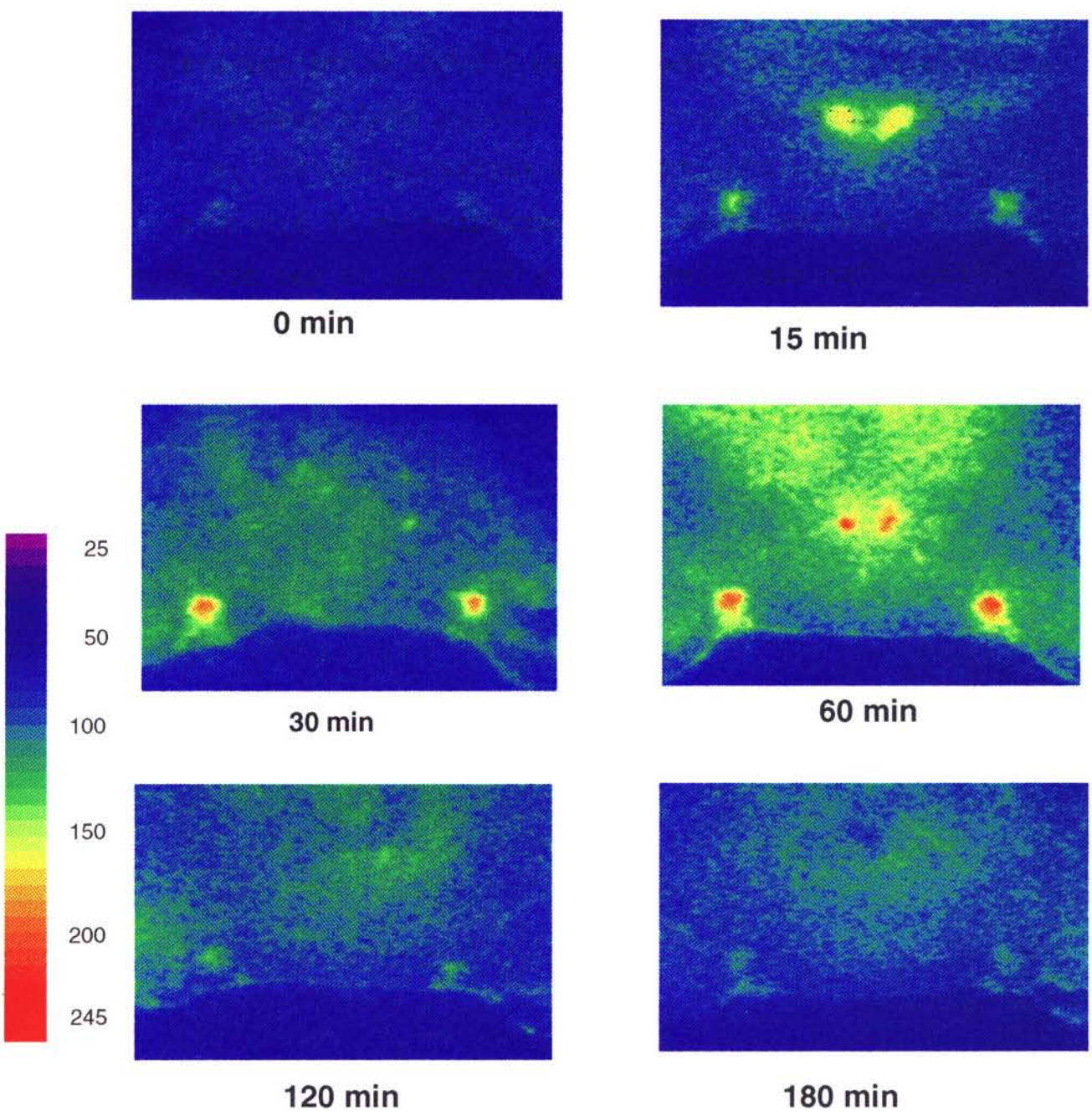


Figure 21. Coronal brain autoradiograms from c-*fos* and c-*jun* mRNA *in situ* hybridization. **2XNS(3hr-30')** Rats received a normal saline injection and were then returned to their home cage for 3hr before receiving the second saline injection. These rats were then sacrificed 30 min later. **1XHS (30')** Rats were used as positive control group, receiving a 1.5 M hypertonic injection and a 30 min survival time. **NS-HS (3hr-30')** Rats first received 0.9% saline, and then a 1.5 M hypertonic injection. **2XHS(3hr-30')** Rats received two 1.5 M hypertonic saline injections. The brain images were generated from computer.

c-*fos and* c-*jun* mRNA Expression in Hypothalamus after Repeated Hypertonic Saline Injection

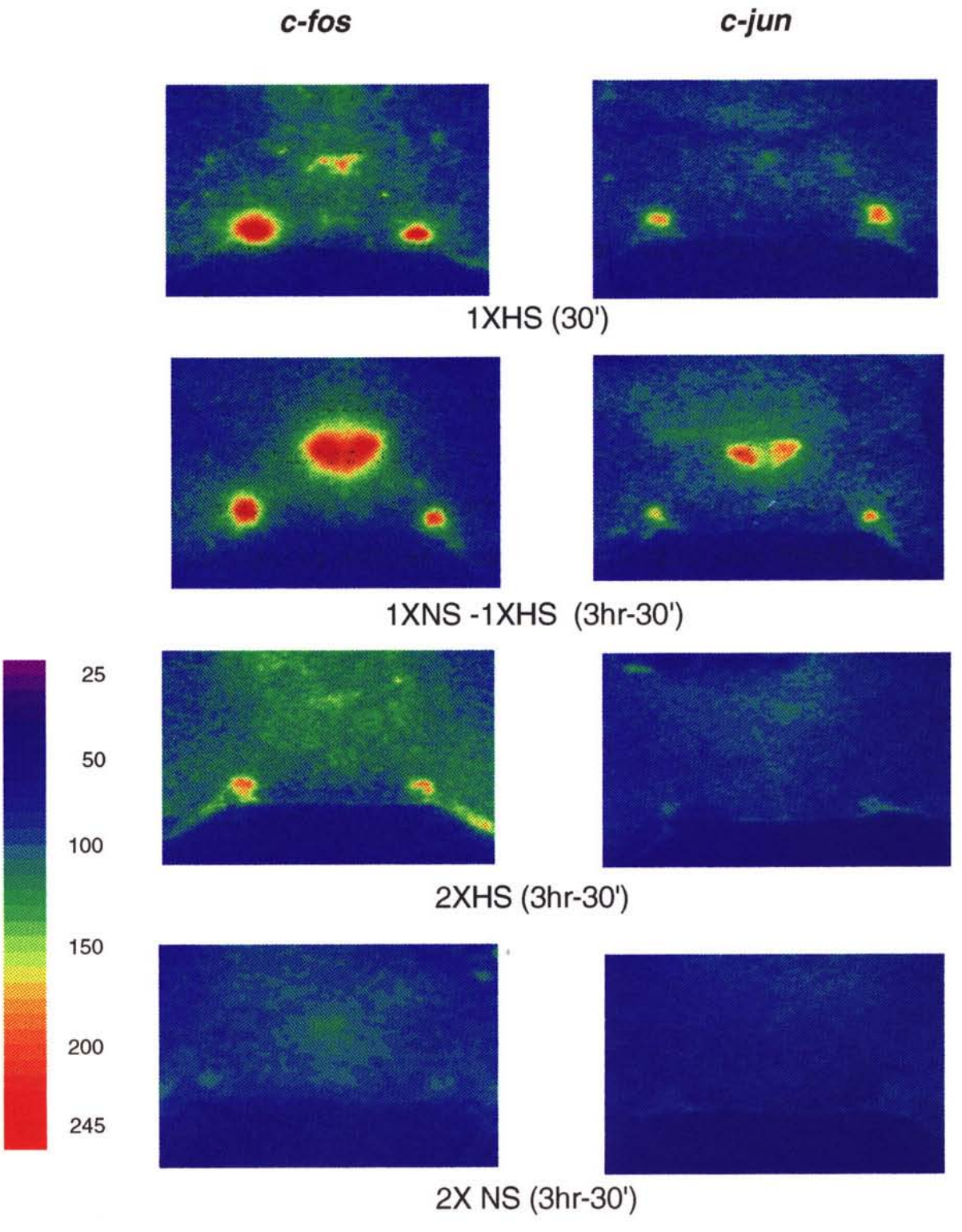
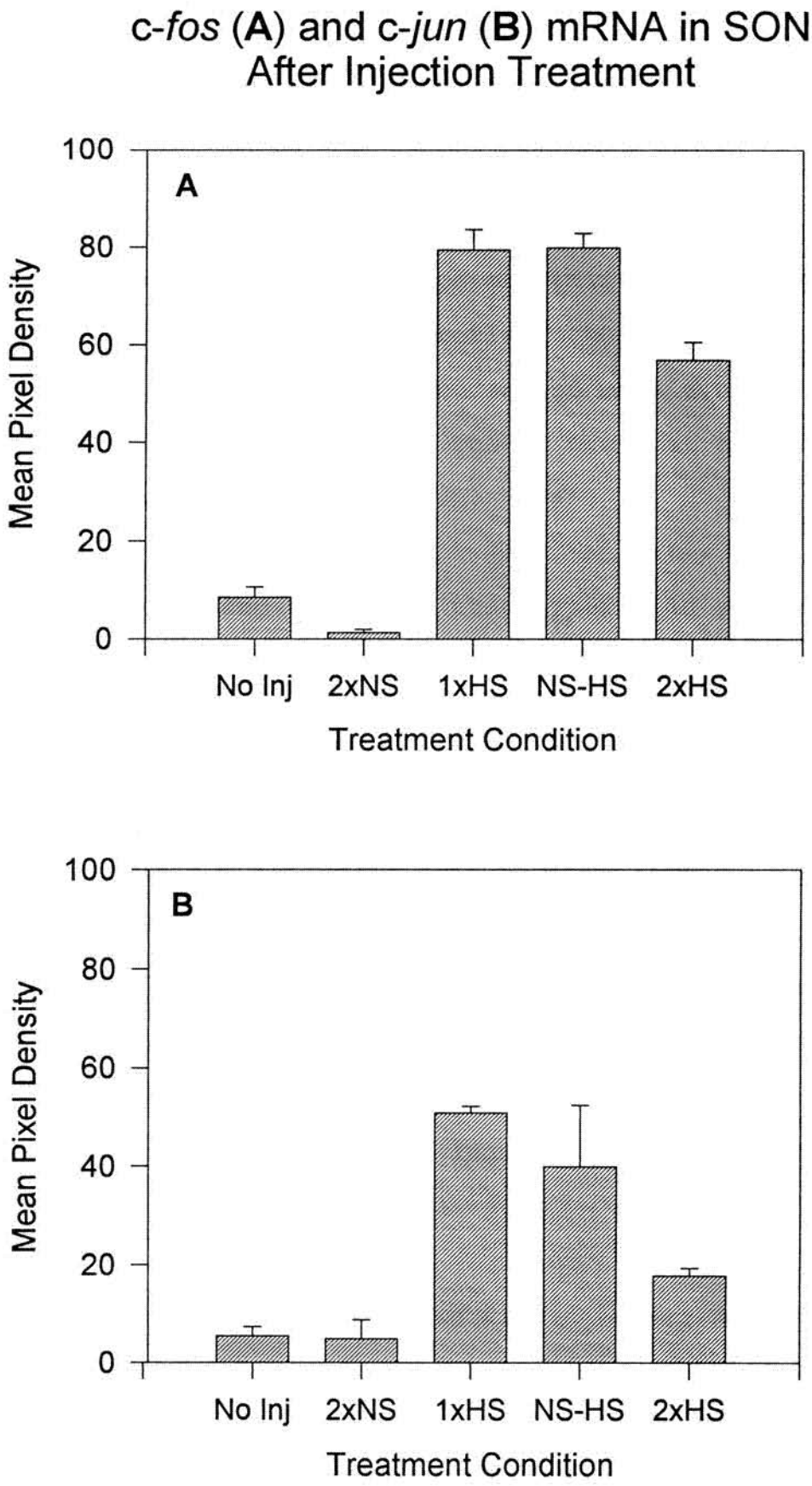


Figure 22. Summary of mean pixel density from hybridization of tissues with probes complementary to c-*fos*(**A**) and c-*jun* (**B**). The mean pixel density of c-*fos* (**A**) for all groups that received one (**1xHS**, **NS-HS**) or two (**HS-HS**) hypertonic saline injections were significantly greater than levels seen in tissue from rats that received no injections (**No Inj**) or two normal saline injections (**2xNS**). Pixel density values for the **HS-HS** group, however, was significantly less than levels in rats that received single hypertonic saline injections (**1xHS**, **NS-HS**: all values $p < 0.05$).

With respect to c-*jun* mRNA levels (**B**), mean pixel density values for the groups that received single injections of hypertonic saline (**1xHS**, **NS-HS**) were significantly greater than the non-injected (**No Inj**) and normal saline injected group (**NS-NS**), as well as greater than levels for the group of rats that received two hypertonic saline injections (**2xHS**). The latter group was not significantly different from the density values seen in tissue from rats that received no injection or normal saline injections.


c-fos (A) and c-jun (B) mRNA in SON
After Injection Treatment
A
B
Mean Pixel Density
100
80
60
40
20
0
No Inj
2xNS
1xHS
NS-HS
2xHS
Treatment Condition

Discussion

In situ hybridization and Northern blotting methods were used to study changes in mRNA levels of c-*fos* and c-*jun* after the induction of plasma hyperosmolality. Although the level of mRNA does not directly reflect the level of gene transcription, since it is independent of mRNA synthesis, stability and degradation, it is one of the most acceptable tools for study of gene expression.

Northern blotting indicated that hypertonic saline administration induced both c-*fos* mRNA and c-*jun* mRNA expression within 5 minutes, that levels peaked 30 to 60 minutes later, and gradually returned to baseline levels by 3 hours (Figure 12). These findings are in agreement with work investigating changes in c-*fos* mRNA levels (Carter et al., 1990; Sharp et al., 1991; Hamamura et al., 1992; Ding et al., 1994). The present results show there is apparently coordinate induction of c-*fos* and c-*jun* after stimulation. This pattern has been observed previously in the hippocampus after treatment with the seizure-inducing drug, metrazole (Sonnenberg et al., 1989).

In situ hybridization results are in agreement with northern blotting data, and show that intraperitoneal hypertonic saline injection induced a transient c-*fos* and c-

jun mRNA response that was evident within 15 min, peaked at 30 to 60 min, gradually decreased by 3 hours (Figure 19 and 20).

Results with both northern and *in situ* hybridization work, however, suggest the changes in levels for both oncogenes are equivalent. In comparison to observed changes in the levels of c-*jun* mRNA, c-*fos* mRNA seems to be induced faster and more strongly after saline injection, and the duration of higher c-*fos* mRNA levels lasts for a longer period of time. Immunofluorescent work (at least for single-labeled cells) followed this pattern of dissociation of c-Fos and c-Jun protein expression, but this result may not be the comparable. In this case, data examined the number of single-labeled cells.

After *repeated* hypertonic saline injection, northern blotting and *in situ* hybridization results demonstrated that c-*fos* mRNA expression was not comparable to changes after one time injection. *In situ* hybridization results suggested that c-*jun* mRNA levels are most dramatically different by this treatment, since c-jun mRNA levels from rats that received two hypertonic saline injection (**2XHS**) failed to increase to levels significantly from the control groups (**No Inj** and **2XNS** groups, Figure 22). These findings are consistent with my protein work where difference between the total number of neurons with Jun immunostaining was more

affected by repeated hypertonic saline treatment (Figure 8). In short, the present results suggest the down-regulation of c-Fos and c-Jun protein is a reflection of what occurs at the transcriptional levels: not only do changes in Fos and Jun protein levels act in a coordinate fashion, but mRNA levels exhibit a similar pattern.

In situ hybridization was employed to survey other brain regions. This work demonstrated that not only magnocellular neurons of SON and PVN increase their expression c-*fos* mRNA after hypertonic saline injection, but also components of the "osmoreceptive complex": the subfornical organ, the median preoptic nucleus, and the organum vasculosum of lamina terminalis. These data are in agreement with my immunocytochemistry findings. Positive signal was also observed in the bed nucleus of the stria terminalis, the parvocellular segment of the PVN, and the nucleus of circularis. These areas are thought to mediate neurological components of drinking behavior (Iovino et al., 1985) and perhaps it is significant that these regions have reciprocal connections with the circumventricular organs, paraventricular nucleus (PVN) and supraoptic nucleus (SON: Phillps et al., 1987). The increased mRNA levels seen in the parvocelluar regions of the PVN may be significant, since these are involved in stress-induced secretion of adrenocorticotropic hormone (ACTH) following hypertonic saline injection (Lightman et al., 1987).

CHAPTER 4

AP-1 DNA-Binding Activity in SON Neurons of Rats Following Fluid Imbalance

Introduction

Research in the late 1980's led to the exciting finding that the transcription factor AP-1 is a complex made up of several proteins, including members of the c-*fos* and c-*jun* gene families (Curran & Franza, 1988). As noted earlier, c-*fos* and c-*jun* protein products can form heterodimers (Fos-Jun and Jun-Jun family member heterodimers) and homodimers (Jun family members) that bind with high affinity to the DNA consensus sequence, AP-1 (Franza et al., 1988; Rauscher et al., 1988c and Gentz et al., 1989). Since the AP-1 regulatory element is known to be essential for the transcription of certain genes (Lee et al., 1987a,b; Angel et al., 1987; Piette and Yaniv, 1987; Distel et al., 1987; Franza et al., 1988; Rauscher et al., 1988a,c), Fos and Jun dimerization and sequence-specific DNA binding represents a crucial link between cell stimulation and subsequent alterations in gene expression. Based upon the profound changes in mRNA levels and protein products in the SON after hypertonic saline injection (Giovannelli et al., 1992;

Roberts et al., 1993, Ding et al., 1994; Chapters 2 and 3), I next attempted to study AP-1 DNA binding activity. Using the gel shift DNA binding assay, AP-1-like DNA binding activity was determined in SON tissue from rats with and without fluid imbalance. This work determined that AP-1-like complex formation occurs under stimulated conditions, and thereby reinforces the idea that actual protein complex formation occurs and makes it possible that these complexes are capable of interaction with the AP-1 binding site consensus sequence *in vivo*.

Materials and Methods

Animal Treatment

Twelve male, Sprague-Dawley rats received a 1.5 M hypertonic injection and were sacrificed 90 minutes later. An additional four rats were used as a control group and received no injection.

Preparation of SON Nuclear Extracts

After experimental treatment, the rats were deeply

anesthetized and then decapitated. The brain was removed from the skull and positioned in a custom mold. Coronal sections containing the entire SON were immediately isolated with the aid of a clean razor blade. The slices (1 mm thickness) were placed on a flat surface cooled with dry ice, and tissue containing the SON was punched, quickly frozen in dry ice, and stored at -70°C. A 16-gauge blunt needle (Becton, Dickinson and Company, Parsippany, NJ) was used for extraction of the SON.

Nuclear enriched extracts were made using a modification of the method of Sonnenberg et al. (1989). Tissue was pooled in a 1.5 ml tube and minced in a 500 μl solution of ice-cold buffer containing 0.25 M sucrose, 15 mM NaCl, 5mM EDTA, 1 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, and protease inhibitors (1mM dithiohreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 5 μg/ml aprotinin). The homogenate was centrifuged at 2000 x g for 10 minutes, the supernatant was discarded, and the pellet was resuspended in 500 μl of buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, and protease inhibitors, and then pelleted by centrifugation at 4,000 x g for 10 minutes. The pellet was resuspended in buffer (0.5 M HEPES, pH 7.9, 0.75 mM EDTA, 0.5 M KCl, 12.5% glycerol plus protease inhibitors) and incubated on a rotator for 30 minutes. After 30 minutes of incubation, cell extracts were

collected by centrifugation at 14,000 x g for 30 minutes. The entire procedure was performed at 4°C. Protein concentration was determined (Bio-Rad Protein Assay; Bio-Rad, Hercules, CA) and ranged from 0.25 to 0.50 $\mu g/\mu l$. Extracts were frozen in liquid nitrogen and stored at -70°C.

Labeling of Consensus Oligonucleotides

Double-stranded oligonucleotides, including the consensus AP-1 site, a mutated Ap-1, and a Sp1 consensus oligo were obtained from Santa Cruz (Santa Cruz Biotech., Santa Cruz, CA) or Promega (Madison, WI). The AP-1 oligonucleotide was radiolabeled by phosphorylation using the Promega Gel Shift Assay System Kit: 2 μl of AP-1 consensus oligonucleotide (1.75pmol/μl), 1 μl of 10X T_4 polynucleotide kinase buffer, 1 μl of [γ^{32}P] ATP (3,000Ci/mmol at 10 mCi/ml), 5 μl of dH_2O, and 1 μl of T_4 polynucleotide kinase were mixed and incubated at 37°C for 10 minutes. The reaction was stopped by adding 1 μl of 0.5 M EDTA and 89 μl of TE buffer. The labeled probes were stored at -20°C until they were used.

Two AP-1 oligonucleotides, from Santa Cruz and Promega, were radiolabeled and tested for their binding to tissue

extracts (see table below). It was noticed that the sequences from the two vendors are different. The consensus binding sites, however, are the same for each and both appeared to produce similar results (the difference in the sequences are indicated by italics). The sequence synthesized by Santa Cruz is correct (see Lee et al., 1987b).

Gel Mobility Shift Assay

A 4% nondenaturing acrylamide gel was made by mixing 5 ml of 10X TBE buffer, 10 ml of 40% Bis and acrylamide (1:29), 3.1 ml of 80% glycerol, 81 ml of dH_20, 50 μl TEMED (N,N,N'N',-tetramethyl-ethylenediamine), and 750 μl of fresh prepared 10% ammonium persulphate. Before loading the samples, the gel was pre-run for 90 minutes at 4°C (Bio-Rad Protean IIxi Cell vertical gel apparatus, Bio-Rad Laboratories, Hercules, CA).

The binding reactions were performed by incubating 5 μg of SON extract or 10 μg of Hela cell nuclear extract (positive control) with 5X gel-shift binding buffer [0.05 mg/ml poly(dI-dC).poly(dI-dC), 25 mM dithiothreitol, 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 250 mM NaCl, and 50 mM Tris-HCl] for 10 minutes at room temperature. One μl of ^{32}P-

labeled AP-1 consensus oligo was then added, and the reactions were incubated at room temperature for an additional 30 minutes. In order to further determine the constituents of the AP-1 protein complex, some sample extracts were incubated (4°C) with 1-2 μl of polyclonal anti-c-Fos [c-*fos*-(4):sc-52] or anti-c-Jun [c-*jun*/AP-1 (D):sc-44] antisera (Santa Cruz Inc., Santa Cruz, CA) for 60 minutes before binding reactions were carried out. The Fos antibody is directed against the amino terminal domain of Fos (amino acids 3-16 of the human c-*fos* p62 protein), and the Jun antibody against Jun's conserved DNA binding domain (residues 247-263 of mouse c-*jun* p39 protein).

Several control experiments were conducted in parallel to ensure specificity of DNA binding. Some samples were incubated as described above, but with the addition of an excess (1 μl) of "cold" AP-1 oligomer (Promega E3201), "cold" mutated AP-1 (20 ng/μl, Santa Cruz, sc-2514), or Sp1 (1.75pmol/μl; Promega E3231) oligonucleotide consensus sequences. The addition of 1 μl of cold oligomers is approximately a 100-fold excess of the amount of radiolabeled AP-1 DNA added to the reaction mixture. In Table 3, the sequences are outlined for each of these oligonucleotides and the protein binding regions are indicated in bold characters. The sequence encoding the "mutation" for the mutated AP-1 oligomer is underlined. It

is expected that incubation with the cold AP-1 oligomer will block all cell extract binding to the radiolabeled oligomer, while incubation with the mutated AP-1 or the Sp1 sequences will not inhibit DNA-protein binding.

Protein-probe complexes were resolved by electrophoresis through 4% nondenaturing polyacrylamide gels in 0.5 x Tris borate buffer (1x buffer: 89mM Tris, 89mM boric acid, 2mM EDTA) at 200 V for 3 hours at room temperature. Gels were dried and exposed to XAR-5 X-ray film (Eastman Kodak, Rochester, NY) for 6-24 hours at -70°C.

Table 3

Summary of Oligonucleotides Used in Gel Shift Assay

Oligomer	Sequence	Vendor No.
Promega AP-1	5′-CGC TTG **ATG AGT CA**G CCG GAA-3′ 3′-GCG AAC T**AC TCA GT**C GGC CTT-5′	E-3201
Santa Cruz AP-1	5′-CGC TTG **ATG ACT CA**G CCG GAA-3′ 3′-GCG AAC T**AC TGA GT**C GGC CTT-5′	sc-2501
Santa Cruz Mutated AP-1	5′-CGC TTG **ATG ACT** TG**G** CCG GAA-3′ 3′-GCG AAC T**AC TGA** AC**C** GGC CTT-5	sc-2514
Promega Sp-1	5′-ATT CGA TC**G GGG CGG GGC** GAG C-3′ 3′-TAA GCT AG**C CCC GCC CCG** CTC G-5	E-3231

Results

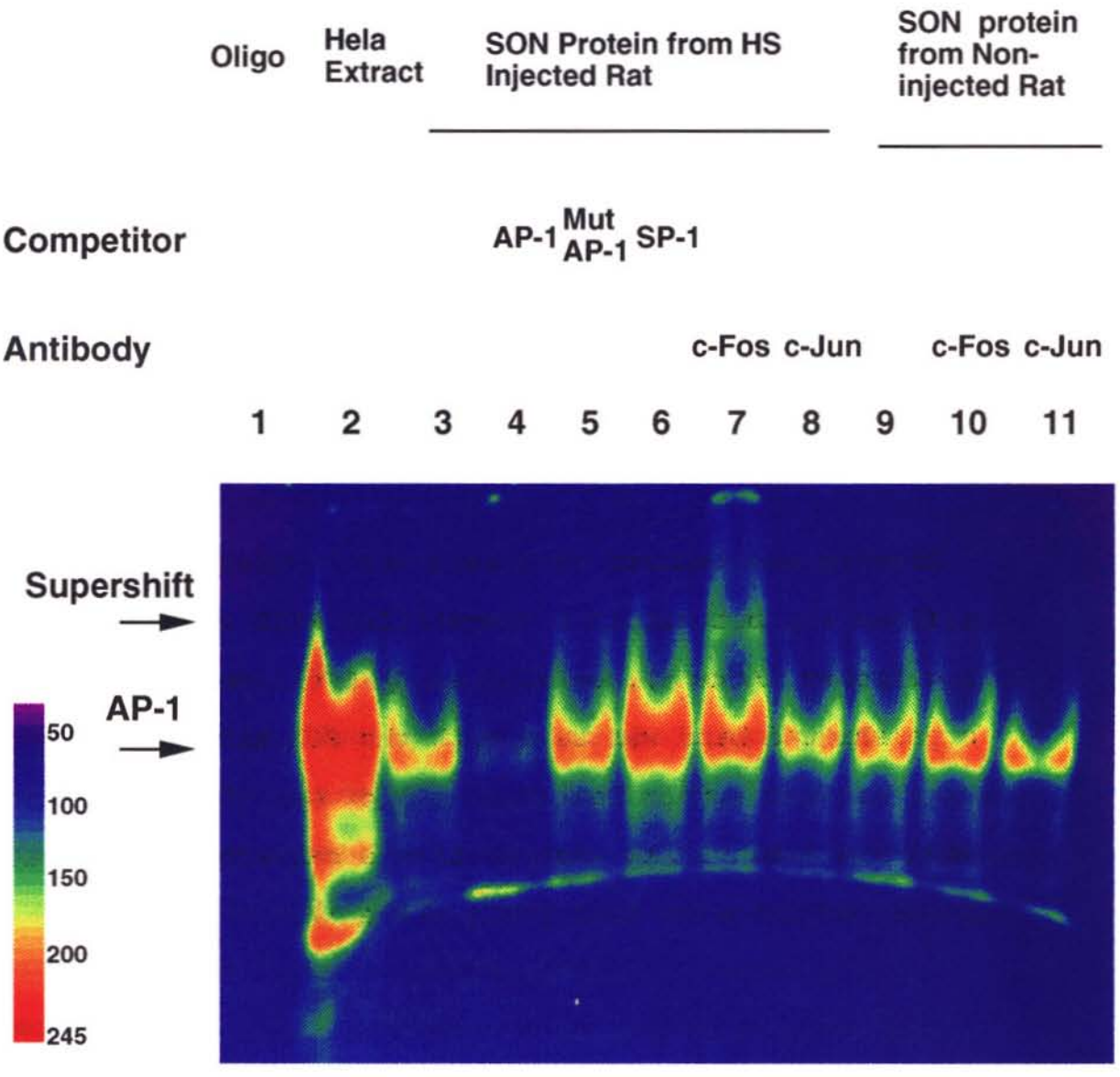
Immunocytochemical analysis of Fos and Jun expression in SON neurons after hypertonic saline administration indicates the staining for these proteins peaks 1.5-2 hours after injection. Using the gel shift assay, I determined whether this was reflected by a similar increase in AP-1 DNA binding activity. SON tissue samples obtained from control (no injection) and hypertonic saline-stimulated groups contained a significant level of AP-1-like DNA binding activity (indicated by AP-1) and the observed band was equivalent to what was seen from incubation with extracts from HeLa cells (Figure 23, lane 2). As expected, a band was not seen when no SON extract was added to the binding reaction mixture (lane 1). The specificity of SON nuclear protein binding to AP-1 was confirmed by competition with unlabeled AP-1, mutated AP-1, and Sp-1 oligonucleotides. As shown in Figure 23, SON nuclear protein binding to labeled AP-1 (lanes 3 and 9) was abolished by incubating the samples with an excess of unlabeled AP-1 (lane 4) but not by incubation with either mutated AP-1 oligonucleotides (lane 5) or unlabeled Sp-1 (lane 6). In addition, preincubation of SON extracts from osmotically stimulated animals with c-Fos antibody resulted in a supershifted band (lane 7). Incubation of samples with the Jun antibody did not cause a

supershift (lane 8), but partially blocked DNA-protein binding (compare lanes 3 and 8, lanes 9 and 11). In tissue samples from non-injected rats, no Fos antibody-supershifted band was observed (lane 10) and there was a partial diminution of binding when samples were incubated with the Jun antibody (lane 11).

Figure 23. Gel-shift DNA binding assay of tissue extracts from a rat that received a hypertonic saline injection (**Lane 3**) indicated the AP-1 oligomer was able to bind to SON neuron extracts, and the band was of a size similar to that from a HeLa cell extract (**Lane 2**). Extracts from a rat that received no saline injection (**Lane 9**), also binds the AP-1 oligomer. Incubation of SON cell extract from the injected rat (**Lane 7**) with an antibody to Fos resulted in a supershift band, while an anti-Jun incubation (**Lane 8**) did not produce a supershift.

Binding was reduced if tissue was incubated with *cold* AP-1 (**Lane 4**), but not in a competition with either *mutated* AP-1 oligomer (**Lane 5**), or with an Sp-1 oligomer (**Lane 6**). No supershifted band was seen with the SON extracts from the control rat for either Fos (Lane 10) or Jun (Lane 11), and no band was seen at all when no tissue extract was incubated with the radiolabeled AP-1 probe (Lane 1).

GEL SHIFT DNA BINDING ASSAY



Discussion

The present findings indicate SON tissue samples contain a protein complex capable of binding radiolabeled oligonucleotides encoding the AP-1 binding site moiety. Several control studies from this experiment support the idea that the binding is specific to an AP-1 or closely related AP-1-like sequence, since 1) SON tissue samples formed a DNA-protein complex similar in molecular weight to a complex formed with HeLa cell extracts, 2) incubation with an excess of cold AP-1 oligomer abolished binding to SON samples, but incubation of samples with excess mutated AP-1 oligonucleotide or Sp1 sequences did not reduce binding, and 3) preincubation with a Fos antibody resulted in the appearance of a supershifted band in tissue samples from a rat that received a hypertonic saline injection. Incubation of samples with the Jun antibody, on the other hand, partially reduced the size of the band. The reason for the difference between results from incubation with the Fos and Jun antibodies is related to the specificity of the epitope binding site for these antisera. The Fos antibody is directed against part of the protein region of the Fos molecule that contains the leucine zipper, and by this coupling it "retards" DNA-protein migration through the gel. The Jun antibody had a different effect where it reduced

DNA-protein binding because the antibody is directed against the DNA binding region of Jun. It thereby "blocks" Jun binding of the tissue protein complex extract to the AP-1 oligonucleotide.

The size of the supershifted band was smaller than the AP-1 band. There are two reasons why these results are less dramatic than, for example, what was observed in the immunocytochemistry experiments where immunostaining to both Fos and Jun was robust. First, the antisera for Fos and Jun that was utilized for immunocytochemistry were polyclonal antibodies which were not specific to just Fos and Jun, respectively, but have potential epitope binding sites to related Fos- and Jun-like proteins. Santa Cruz indicates their antisera for gel shift assays are very specific to Fos and Jun and do not interact with other members of the Fos and Jun families. Secondly, we have learned through communication with Santa Cruz that they have observed stronger binding of antibody to the antigens, and a stronger supershift band, when the tissue extracts are incubated with the radiolabeled oligonucleotide before adding the antisera. In the present work I had incubated the tissue extracts and antibodies first, and then added the oligonucleotides.

The present gel shift data suggests there are protein complexes in the SON of non-injected rats that can couple to

the AP-1 DNA binding site. The data is similar to reports by Sonnenberg and colleagues (1989). These authors suggested that a significant level of AP-1-like DNA binding activity in control rats (non-treatment) could be Fra. Fos-related proteins (FosB, Fra-1, Fra-2) and Jun-related proteins (JunB, JunD) can all give rise to AP-1 binding in combination with Fos and Jun.

Results from my experiment did not indicate a significant difference in the size of the AP-1 complex bands for hypertonic saline injected and non-injected rats. I was surprised by this result. I hypothesized that the dramatic increase in Fos and Jun immunostaining I observed in SON tissue from hypertonic saline administration, compared to nonexistent staining in non-injected rats, would have been reflected in a much stronger band in the gel (compare lanes 7 and 8 in Figure 23; the latter lane is the tissue sample from the non-injected rat).

There may be several explanations for this result, but the simplest may be that there are a equivalent levels of other DNA binding proteins already present in SON tissue, and that these bind to the AP-1 oligomer. The protein CREB, for example, is expressed constitutively in many tissues and this protein may bind to the AP-1 sequence, which differs from the CRE sequence by a single base (Delmas

et al., 1994). Recent work by Mellström and colleagues (1993), however, shows that salt water administration does not induce CREB in the SON, and they report CREB is not expressed in the SON. Saline administration does increase mRNA levels for the cAMP-response element modulators (CREMs), CREMα and CREMß. These factors act as antagonists of CRE-stimulated transcription. However, the mRNAs for CREMα and ß do not appear in the SON until 3 hours post-injection, a time course that eliminates them as factor able to bind to the AP-1 oligonucleotide. Finally, members of the ATF family bind to the consensus site 5'-GTGACGT$^{A}_{C}{}^{A}_{G}$-3' which differs by a single base from the AP-1 site (Chatton et al., 1994; Hai et al., 1989 and 1991). ATF proteins can form heterodimers with Fos and Jun (Chatton et al., 1994) and ATF-2 mRNA is present in brain tissue (Maekawa et al., 1989). No work has been done, however, to determine which anatomical brain regions express this message.

The results from a comparison of SON tissue samples from stimulated (hypertonic saline injected) and nonstimulated rats did, however, exhibit a difference when tissue extracts were incubated with antibody to Fos. My results suggested a difference in DNA binding activity inasmuch as the sample from stimulated rats resulted in a supershifted band and a strong blocking effect. These findings are in agreement with the previous results where I

found the hypertonic saline stimulus induced c-Fos and c-Jun expression, while little Fos or Jun immunoreactivity was seen in SON samples from rats that received no injection or normal saline.

Further DNA binding assay work should be undertaken where binding to SON tissue is tested not only with an AP-1 sequence-specific oligonucleotide, but with sequences for the canonical CRE sequences. Supershift assays with antisera specific to additional members of the Fos, Jun, and CREB/CREM-ATF families should also be assessed to determine if there is differential activity in stimulated and unstimulated animals.

Chapter. 5

Summary

I have investigated c-Fos/c-*fos* and c-Jun/c-*jun* expression in neurons of the SON after acute and repeated hypertonic saline injection. This is a valuable experimental paradigm for investigation because the supraoptic nucleus is a major component of the neuroendocrine system that mediates body water balance. These neurons, *via* their axonal projections to the posterior pituitary, secrete vasopressin (anti-diuretic hormone) into the circulation. Secretion of this hormone is under close regulatory control, since physiological experiments show even small, incremental changes in blood osmolality cause proportionate changes in plasma hormone concentration. Thus, the control of release of hormone, a function of the neuronal firing activity of SON and PVN neurons, is able to tonically modulate the rate of water reabsorption by the kidney.

My investigations, as well as the work of others, indicates there are very dramatic increases in Fos and Jun expression after acute changes in plasma osmolality. Fluorescent immunocytochemistry, northern blotting, and *in*

situ hybridization demonstrated that after acute hypertonic saline injection, Fos/c-*fos* and Jun/c-*jun* levels increased, from previously undetectable levels, in the majority of SON neurons. In comparison, a few scattered Fos/c-*fos* and Jun/c-*jun* nuclei were found if rats received an isotonic saline-injection. The temporal change in levels of Fos/c-*fos* and Jun/c-*jun* appears to occur in a coordinated manner. I have no findings to explain how this may occur, but must assume it was not a coincidental phenomenon, and that this similar change in mRNA and protein levels for these transcription factors indicates they have a significant physiological role in cellular response, and that (as shown in many *in vitro* studies) these proteins interact.

The results of counting the proportion of neurons with immunostaining for Fos and Jun suggested that, after osmotic stimulation, 20% of SON neurons showed both Fos and Jun immunostaining at 30 minutes, 80% at 90 to 120 minutes, and less than 20% at 4 hours. A few neurons appeared to have expressed either c-Fos or c-Jun protein. As might be expected, changes in mRNA levels preceded changes for the proteins. *In situ* hybridization and Northern blotting found both c-*fos* and c-*jun* expression after hypertonic injection was induced within 5 min, peaked at 30 to 60 min, and gradually disappeared by 3 hr. There were differences in the rapidity and degree of induction following stimulation.

c-*fos* mRNA levels seemed to increase sooner and to higher levels than c-*jun*, and higher levels appeared to last for a longer time. Although my previous immunofluorescent work derived similar results, the magnitude of the differences were more dramatic at the mRNA level.

Administration of hypertonic saline injection caused Fos and Jun immunostaining not only in magnocellular neurons of the SON, but in the PVN, and the "osmoreceptive complex": the subfornical organ (SFO), the median preoptic nucleus (MnPO), and the organum vasculosum of lamina terminalis (OVLT). *In situ* hybridization demonstrated that magnocellular neurons of the PVN and the nucleus circularis expressed both c-*fos* and c-*jun* mRNA. In contrast, in the "osmoreceptive complex", and anatomical regions known to be associated with drinking behavior (the bed nucleus of the stria terminals, and the parvocellular part of PVN), c-*fos* mRNA expression, but not c-*jun*, was significantly increased after hypertonic saline treatment.

Following repeated hypertonic saline injection, the percentage of SON neurons with colocalized c-Fos and c-Jun immunoreactivity (< 20% colocalization) was dramatically less compared with what happened after a single acute injection (>80% colocalization). This occurred in spite of the fact that for both groups osmolality and sodium levels

were not different. Consistent with this protein work, c-*fos* and c-*jun* mRNA expression was also repressed by repeated injections. The comparison of the level of the decrement for each gene product indicated the effect was not identical. The apparent down-regulation of c-Fos/c-*fos* expression was less than that of c-Jun/c-*jun*. The molecular mechanisms causing there to be less response after repeated injections is not known. However, it is known that Fos induces auto-regulation, and Jun-B may play an important role in the regulation of c-Jun expression. This repression of c-*fos* and c-*jun* expression may be an important mechanism for normal cellular survival. *In vitro* gene transfer studies have shown that overexpression of c-*fos* can stimulate the expression of differentiation markers in embryonal carcinoma cells (Müller et al., 1984; and Rüther et al., 1985). Analogously, it is known that Jun induces cancer through its role as a transcriptional regulator (for review, see Peter et al., 1990). Overexpression of some of these genes could conceivably contribute to the malignant cellular phenotype (Fujita et al., 1986; Angel et al., 1987).

Gel shift assay showed that SON extracts incubated with a specific c-Fos antibody can form a supershift band in hypertonic saline injected rats, but not control (noninjected) rats. Taken together, these data support the

idea that hypertonic saline injection induces c-Fos and c-Jun protein expression, that Fos and Jun can bind to AP-1 DNA binding sites, and that these changes may be involved in osmotic regulation. Osmotic challenges from salt water drinking not only induces the most important gene for body water regulation (vasopressin) in SON neurons but several other genes. Salt water drinking increases mRNA levels encoding dynorphin, enkephalin, and tyrosine hydroxylase, and all these genes contain an AP-1 sequence in their 5'-regions (Gizeng-Ginsberg et al., 1990; Naranjo et al., 1991). These genes may all be activated by increased AP-1 DNA binding activity after osmotic challenge. Since the promoter region of the vasopressin gene contains a putative CRE binding site (Mohr et al., 1990), further work should be undertaken to determine if CRE binding activity is also increased after hypertonic saline administration.

In conclusion, c-*fos* and c-*jun* levels are transiently changed in a coordinate manner after acute hypertonic saline injection. The appearance, duration, and intensity of changes may be slightly different for these two genes.

Compared with what occurred after acute injection, c-Fos/c-*fos* and c-Jun/c-*jun* levels were dramatically different after repeated injections. The apparent down-regulation

appears to occur at the transcriptional level since the changes observed with immunocytochemistry were also seem at the mRNA level.

Bibliography

Adell, A., Garcia-Marquez, C., Armario, A. and Gelpi, E. 1988. Chronic stress increases serotonin and noradrenaline in rat brain and sensitizes their responses to a further acute stress. *J. Neurochem.* **50**:1678-1681

Altstein, M., Whitnall, M.H., House, S., Key, S. and Gainer, H. 1988. An immunochemical analysis of oxytocin and vasopressin prohormone processing in vivo. *Peptides* **9**: 87-105

Amico, J.A. and Robinson, A.G., (eds) 1985. **Oxytocin.** Amsterdam: Excepta Medica, pp. 1-444

Angel, P. and Karin, M. 1991 The role of Jun, Fos and AP-1 complex in cell-proliferation and transformation. *Biochem. Biophys. Acta* **1072**: 129-157

Angel, P., Allegretto, E.A., Okino, S.T., Hattori, K., Boyle, W.J, Hunter, T., Karin, M. 1988. Oncogene jun encodes a sequence-specific trans-activator similar to AP-1. *Nature* **332**: 166-171

Angel, P., Imagawa, M., Chiu, R., Imbra, R.J., Rahmsdorf, H.J., Herrlich, P. 1987. Phorbol-ester-inducible genes contain a common *cis*-element recognized by a TPA-modulated trans-activating factor. *Cell* **49**: 729-739

Bartel, D.P., Sheng, M., Lau, L.F., and Greenberg, M.E. 1989. Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of fos and jun induction. *Genes Dev.* **3**: 304-313

Barzilai, A., Kennedy, T.E., Sweatt, J.D., and Kandel, E.R. 1989. 5-HT modulates protein synthesis and the expression of specific proteins during long-term facilitation in Aplysia sensory neurons. *Neuron* **2**: 1577-1586

Berne, R.M and Levy, M.N. (1988) **Physiology, Second Edition.** St. Louis, C.V. Mosby Company, pp. 794-797.

Bishop, J.M. 1983. Cellular oncogenes and retroviruses. *Annu. Rev. Biochem.* **52**: 3013-54

Bishop, J.M. 1987. The molecular genetics of cancer. *Science* **235**: 305-310

Bishop, J.M., Varmus, H.E. 1982. Functions and origins of retroviral transforming genes. ed. R. Weiss et al, pp. 999-1108. *New York: Cold Spring Harbor Lab.* **2nd ed.**

Black, I.B., Adler, J.E., Dreyfuss, C.F., Friedman, W.F., La Gamma, E.F., Roach, A.H. 1987. Biochemistry of information storage in the nervous system. *Science* **236**: 1263-1268

Blanar, M.A., and Rutter W.J. 1992. Interaction cloning: identification of a Helix-Loop-Helix Zipper protein that interacts with c-Fos. *Science* **256**: 1014-1018

Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K.,
Tijian, R. 1987. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. *Science* **238**: 1386-1392

Bourque, C.W. 1989. Ionic basis for the intrinsic activation of rat supraoptic neurones by hyperosmotic stimuli. *J. Physiol.* **417**:263-277

Brownstein, M.J. 1983. Biosynthesis of vasopressin and oxytocin. *Annu. Rev. Physiol.* **45**: 129-135

Brownstein, M.J. and Mezey, É. 1986. Multiple chemical messengers in hypothalamic magnocellular neurons. *Prog. Brain Res.* **68**: 161-168

Carter, D.A., and Murphy,D. 1990. Regulation of c-fos and c-jun expression in the rat supraoptic nucleus. *Cell. Mol. Neurobio.* **10**: 435-445

Castel, M., Gainer, H., Dellmann, H.D. 1984. Neuronal secretory systems. *International Rev. Cytology* **88**: 304-459

Castellucci, V.F., Kennedy, T.E., Kandel, E.R., and Goelet, P. 1988. A quantitative analysis of 2-D gels identifies proteins in which labeling is increased following long-term sensitization in Aplysia. *Neuron* **1**: 321-328

Ceccatelli, S., Villar, M.J., Goldstein, M., and Hökfelt, T. 1989. Expression of c-fos immunoreactivity in transmitter-characterized neurons after stress. *Proc. Natl. Acad. Sci. USA* **86**: 9569-9573

Chatton, B., Bocco, J.L., Goetz, J., Gaire, M., Lutz, Y., and Kedinger, C. 1994. Jun and Fos heterodimerize with ATFa, a member of the ATF/CREB family and modulate its transcriptional activity. *Oncogene* **9**: 375-385

Chaudhry, M.A., Dyball, R.E.J., Honda, K., and Wright, N.C. 1989. The role of interconnection between supraoptic nucleus and anterior third ventricular region in osmoregulation in the rat. *J Physiol.* **410**: 123-135

Chiu, R., Angel, P., Karin, M. 1989. *Jun*-B differs in its biological properties from, and is a negative regulator, of c-*jun*. *Cell* **59**: 979-986

Cohen, D.R. and Curran, T. 1988. *fra*-1: A serum inducible, cellular immediate-early gene that encodes a Fos related antigen. *Mol. Cell. Biology.* **8**: 2063-2069

Cohen, D.R. and Curran, T. 1989. The structure and function o the *fos* protooncogene. *Crit. Rev. Oncogenesis* **1**: 65-88

Cohen, D.R. and Ferreira, P.C.P., Gentz, R., Franza, B.R.Jr., and Curran, T. 1989. The product of a Fos-related gene, fra-1, binds cooperatively to the AP-1 site with Jun: Transcription factor AP-1 is comprised of multiple protein complexes. *Genes Dev.* **3**: 173-84

Comb, M., Brinberg, N.C., Seasholtz, A., Herbert, E., and Goodman, H.M. 1986. A cyclic AMP and phorbol ester-inducible DNA element. *Nature* **323**: 353-397

Curran, T. 1988. The *fos* oncogene. In **The Oncogene Handbook**, E.P. Reddy ed., Amsterdam: Elsevier, pp. 307-325

Curran, T. and Franza, Jr. B.R. 1988. Fos and Jun: The AP-1 connection. *Cell* **55**: 395-97

Curran, T., Abate, C., Cohen D.R., Macgregor P.F., Rauscher F.J., Sonnenberg J.L., Connor J.A., Morgan J.I. 1990. Inducible proto-oncogene transcription factors: Third messengers in the brain? *Cold Spring Harbor Symposia on Quantitative Biology* **55**: 225-34

Curran, T., Gordon, M.B., Rubino, K.L., and Sambucetti, L.C. 1987. Isolation and characterization of the *c-fos* (rat) cDNA and analysis of post-translational modification *in vitro*. *Oncogene* **2**: 79-84

Curran, T. and Morgan, J.I. 1987. Memories of fos. *BioEssays* **7**:255-258

Curran, T. and Teich, N.M. 1982. Candidate product of the FBJ murine osteosarcoma virus oncogene: Characterization of a 55,000 dalton phosphoprotein. *J. Virol.* **42**: 114-22

Delmas, V., Molina, C.A., Lalli, E., De Groot, R., Foulkes, N.S., Masquilier, D., and Sassone-Corsi, P. 1994. Complexity and versatility of the transcriptional response to cAMP. *Rev. Phyiol. Biochem. Pharmacol.* **124**: 2-23

Ding, J.M., Carver W.C., Terracio, L. and Buggy, J. 1994. Proto-oncogene c-*fos* and the regulation of vasopressin gene expression during dehydration. *Mol. Brain Res.* **21**:247-255

Dhawan, R.R and Beebe, D.C. 1994. Differential localization of collagen typeIX isoform messenger RNAs during early ocular development. *Invest. Ophthalmol. Vis. Sci.* **35**:470-478

Distel, R.J., Ro, H.S., Rosen, B.S., Groves, D.L., Spiegelman, B.M. 1987. Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: Direct participation of c-fos. *Cell* **49**: 835-844

Doris, P.A. 1984. Vasopressin and central integrative processes. *Neuroendocrinology* **38**: 75-85

Dragunow, M., Robertson, H.A. 1987. Kindling stimulation induces c-fos protein(s) in granule cells of the rat dentate gyrus. *Nature* **329**: 441-442

Dragunow, M., and Faull, R. 1989. The use of c-*fos* as a metabolic marker in neuronal pathway tracing. *J. Neurosci. Methods* **29**: 261-265

Ferguson, A.V. and Renaud, L.P. 1986. Systemic angiotensin acts at subfornical organ to facilitate activity of neurohypophysial neurons. *Am. J. Physiol.* **251**: R712-R171

Fitzsimmons, J.T. 1986. Thirst and sodium appetite. In S.L. Lightman and B.J. Everitt (Eds.), *Neuroendocrinology*, Blackwell, Oxford. pp.207-228

Franza, B.R., Jr., Rauscher, F.J., III., Josephs, S.F., Curran, T. 1988. The Fos complex and Fos-related antigens recognize sequence elements that contain AP-1 binding sites. *Science* **239**: 1150-1153

Franza, B.R., Jr., Sambucetti, L.C., Cohen, D.R. and Curran, T. 1987. Analysis of Fos protein complexes and Fos-related antigens by high-resolution two-dimensional gel electrophoresis. *Oncogene* **15**: 5657-5667

Fujita, T., Shibuya, H., Ohashi, T., Yamanishi, K., and Taniguchi, T. 1986. Regulation of human interleukin-2 gene: functional DNA sequences in the 5′ flanking region for the gene expression in activated T lymphocytes. *Cell* **46**: 401-407

Gainer, H., Russell, J.T. and Loh, Y.P. 1985. The enzymology and intracellular organization of peptide precursor processing: The secretory vesicle hypothesis. *Neuroendocrinology* **40**: 171-184

Ganten, D. and Pfaff, D.W. (eds.) 1985. **Neurobiology of Vasopressin** (*Current Topics in Neurobiology, vol. 4*), Berlin: Springer-Verlag, pp. 1-203

Ganten, D. and Pfaff, D. W. (eds.) 1986. **Neurobiology of Oxytocin** (*Current Topics in Neuroendocrinology, vol. 6*), Berlin: Springer-Verlag, pp. 1-190

Gass, P., Herdegen, T., Bravo, R., and Kiessling, M. 1992. Induction of immediate early gene encoded proteins in the rat hippocampus after bicuculline-induced seizures: differential expression of KROX-24, Fos and Jun proteins. *Neuroscience* **48**: 315-324

Gentz, R., Rauscher, F.J., III., Abate, C. and Curran, T. 1989. Parallel association of Fos and Jun leucine zippers juxtaposes DNA-binding domains. *Science* **243**:1695-1699

Giovannelli, L., Shiromani, P.J., Jirikowski, G.F., and Bloom, F.E. 1990. Oxytocin neurons in the rat hypothalamus exhibit c-fos immunoreactivity upon osmotic stress: *Brain Res.* **531**: 299-303

Giovannelli, L., and Bloom, F.E. 1992a. c-Fos protein expression in the rat subfornical organ following osmotic stimulation. *Neurosci. Lett.* **139**: 1-6

Giovannelli, L., Shiromani, P.J., Jirikowski, G.F., and Bloom, F.E. 1992b. Expression of c-fos protein by immunohistochemically identified oxytocin neurons in the rat hypothalamus upon osmotic stimulation. *Brain Res.* **588**: 41-48

Gizeng-Ginsberg, E. and Ziff, E.B. 1990. Nerve growth factor regulates tyrosine hydroxylase gene transcription through a nucleoprotein complex that contains c-Fos. *Genes Dev.* **4**: 477-491

Goelet, P., Castellucci, V.F., Schacher, S., and Kandel, E.R. 1986. The long and the short of long-term memory-a molecular frame-work. *Nature* **322**: 419-422

Goldman, D., Breener H.R., and Heinemann, S. 1988. Acetylcholine receptor α, ß, r and δ subunit mRNA levels are regulated by muscle activity. *Neuron* **1**: 329-333

Greenberg, M.E., Greene, L.A., and Ziff, E.B. 1985. Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. *J. Biol. Chem.* **260**: 14101-14110

Greep, R.O. and Astwood, E.B.(eds.) 1974. Endocrine Glands. In: **Handbook of Physiology, Section 7: Endocrinology, vol. IV. The Pituitary Gland and Its Neuroendocrine Control, Part 1.** Washington, D. C.: American Physiological Society, pp. 1-576

Guldenaar, S.E.F., Noctor, S.C., McCabe, J.T. 1992. Fos-like immunoreactivity in the brain of homozygous diabetes insipidus Brattleboro and normal Long-Evans rats. *J. Comp. Neurol.* **322**: 439-448

Guldenaar, S.E.F., Wang, K., McCabe, J.T. 1994. Double immunofluorescence staining of Fos and Jun in the hypothalamus of the rat. *Cell Tissue Res.* **276**: 1-6

Hai, T. and Curran, T. 1991. Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. *Proc. Natl. Acad. Sci. USA* **88**: 3720-3724

Hai, T., Liu, F., Coukos, W.J., and Green, M.R. 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. *Gene Dev.* **3**: 2083-2090

Hamamura, M., Nunez, D.J.R., Leng, G., Emson, P.C., and Kiyama, H. 1992. c-fos may code for a common transcription factor within the hypothalamic neural circuits involved in osmoregulation. *Brain Res.* **572**: 42-51

Halazonetis, T.D., Georgopoulos, K., Greenberg, M.E., Leder, P. 1988. c-*jun* dimerizes with itself and with c-Fos, forming complexes of different DNA-binding affinities. *Cell* **55**: 917-924

Hatton, G.I. 1990. Emerging concepts of structure-function dynamics in adult brain: the hypothalamo-neurohypophysial system. *Prog. Neurobil.* **34**: 437-504

Hayward, M.D., Duman, R.S., Nestler, E.J. 1990. Induction of c-fos proto-oncogene during opiate withdrawal in the locus coeruleus and other regions of rat brain. *Brain Res.* **525**: 256-266

Herdegen, T., Kovary, K., Leah, J., and Bravo, R. 1991a. Specific temporal and spatial distribution of JUN, FOS, and KROX-24 proteins in spinal neurons following noxious transsynaptic stimulation. *J. Comp. Neurol.* **313**: 178-191

Herdegen, T., Kummer, W., Fiallos, D.E., Leah, J. and Barvo, R. 1991b. Expression of c-JUN, JUN B and JUN D proteins in rat nervous system following transection of vagus nerve and cervical sympathetic truck. *Neuroscience* **45**: 413-422

Herman, J.P., Schäfer, M.K.-H., Sladek, C.D., Day, R., Young, E.A., Aki, H. and Watson, S.J. 1989. Chronic electroconvulsive shock treatment elicits up-regulation of CRF AND AVP mRNA in select populations of neuroendocrine neurons. *Brain Res.* **501**: 235-246

Hoffman, G.E., Lee, W.S., Arrardi, B., Yann, V., Fitzsimmon, M.D. 1990. Luteinizing hormone-releasing hormone neurons express c-fos antigen after steroid activation. *Endocrinology* **126**: 1736-1741

Honda, K., Negoro, H., Dyball, R.E.J., Higuchi, T. and Takano, S. 1990. The osmoreceptor complex in the rat: evidence for interactions between the supraoptic and other diencephalic nuclei. *J. Phsiol.* **431**: 225-241

Hunt, S.P., Pini, A., Evan, G. 1987. Induction of the c-fos protein in spinal coed neurons following sensory stimulation. *Nature* **328**: 432-434

Hunter, T. and Karin, M. 1992. The regulation of transcription by phosphorylation. *Cell* **70**: 375-387

Imake, T., Nahan, J.L., Rivier, C., Sawchenko, P.E. and Vale, W. 1991. Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. *J. Neurosci.* **11**: 585-599

Insel T.R. 1990. Regional induction of c-fos-like protein in rat brain after estradiol administration. *Endocrinology* **126**: 1849-1853

Iovino, M., and Streado, L. 1985. Thirst and vasopressin secretion following central administration of angiotensin II in rats with lesions of the septal area and subfornical organ. *Neuroscience* **15**: 61-67

Irwin, J., Ahluwalia, P. and Anisman, H. 1986. Sensitization of norepinephrine activity following acute and chronic footshock. *Brain Res.* **379**: 98-103

Ivell, R. and Richter, D. 1984. Structure and comparison of the oxytocin and vasopressin genes from rat. *Proc. Natl. Acad. Sci.* **81**: 2006-2010

Ju, G., Liu, S., and Tao, J. 1986. Projections from the hypothalamus and its adjacent areas to the posterior pituitary. *Neuroscience* **19**: 803-828

Karin, M. 1994. Signal transduction from the cell surface to the nucleus through the phosphorylation of transcription factors. *Curr. Opin. Cell Biol.* **6**: 415-424

Klarsfeld, A., Laufer, R., Ontaine, B., Devillers-Thiery, A., Dubreuil, C., and Changeux, J.P. 1989. Regulation of the muscle AChR a subunit gene expression by electrical activity: involvement of protein kinase C and Ca^{2+}. *Neuron* **2**: 1229-1236

König, H., Ponta, H., Rahmsdorf, U., et al. 1989. Autoregulation of fos: the dyad symetry element as the major target of repression. *EMBO J.* **8**: 2559-2566

Krukoff, T.L. 1994. Expression of c-*fos* in studies of central autonomic and sensory systems. *Mol. Neurobiol.* **7**: 247-263

Lafarga, M., Berciano, M.T., Martinez-Guijarro, F.J., Ander, M.A., Mellström, B., Lopez-Garcia, C., and Naranio, J.R. 1992. Fos-Like expression and nuclear size in osmotically stimulated supraoptic nucleus neurons. *Neuroscience* **50**: 867-875

Lafarga, M., Martinez-Guijarro, F.J., Berciano, M.T., Blasco-Ibañez, J.M., Ander, M.A., Mellström, B., Lopez-Garcia, C., and Naranio, J.R. 1993. Nuclear FOS domains in transcriptionally activated supraoptic nucleus neurons. *Neuroscience* **57**: 353-364

Larson, B. L., Ed. 1985. **Lactation**. Ames: Iowa State University Press, pp. 1-276

Lau, L.F. and D. Nathans. 1987. Expression of a set of growth-related immediate-early genes in BALB/c 3T3 cells: Coordinate regulation with c-fos or c-myc. *Proc. Natil. Acad. Sci. USA* **84**: 1182-86

Lee, W., Haslinger, A., Karin, M. and Tjian, R. 1987a. Two factors that bind and activate the human metallothionein II_A gene *in vitro* also interact with the SV-40 promoter and enhancer regions. *Nature* **325**: 368-372

Lee, W., Mitchell, P. and Tjian, R. 1987b. Purified transcription factor AP-1 interact with the TPA-inducible enhancer elements. *Cell* **49**: 741-752

Lee, K.A.W., Hai, T.Y., Siva Raman, L., et al. 1987c. A cellular protein, activating transcription factor, AP-1, activates transcription of multiple E1A-inducible adenovirus early viral promoters. *Proc. Natl. Acad. Sci. USA* **84**: 8355-8359

Lee, K.A.W., Fink, J.S., Goodman, R.H. and Green, M.R. 1989. Distinguishable promoter elements containing ATF-binding sites are involved in transcriptional activation by E1A and cAMP. *Mol. Cell Biol.* **9**: 4390-4397

Leng, G., Dyball, R.E.J., and Mason, W.T. 1985. Electrophysiology of osmoreceptors. In R.W. Schrier (Ed.), *Vasopressin*, Raven, New York, pp.333-342

Leng, G., Mason, W.T. and Dyer R.G. 1982. The supraoptic nucleus as an osmoreceptor, *Neuroendocrinology*. **34**: 75-82.

Leng, F., Blackbrun, R.E., Dyball, R.E.J. and Russell, J.A. 1989. Role of anterior peri-third ventricular structures in the regulation of supraoptic neuronal activity and neurohypophysial hormone secretion in the rat. *J. Neuroendocrinology*. **1**: 35-46

Lightman, S.L. and Young III, W.S. 1987. Vasopressin, oxytocin, dynorphin and corticotrophin-releasing factor mRNA stimulation in the rat. *J. Physiol*. **394**: 23-39

Lucibello, F.C., Lowag, C., Neuberg, M., Müller, R. 1989. Trans-repression of the mouse c-fos promoter: a novel mechanism of Fos-mediated trans-regulation. *Cell* **59**: 999-1007

Maekawa, T., Sakura, H., Kanei-Ishii, C., Sudo, T., Yoshimura, T., Fujisawa, J., Yosshida, M., and Ishii, S. 1989. Leucine zipper structure of the protein CRE-BP1 binding to the cyclic AMP response element in brain. *EMBO. J.* **8**: 2023-2028

Maki, Y., Bos, T.J., Davis, C., Starbuck, M., and Vogt, P.K. 1987. Avian sarcoma virus 17 carries a new oncogene, jun. *Proc. Natl. Acad. Sci.* **84**: 2848-52

Mamalake, E., Kvetnansky, R., Brady, L.S., Gold, P.W. and Herkenham, M. 1993. Repeated immobilization stress alters tyrosine hydroxylase, corticotropin-releasing hormone and corticosteroid receptor messenger ribonucleic acid levels in rat brain. *J. Neuroendocrinology.* **4**: 689-699

Mason, W.T. 1980. Supraoptic neurones of rat hypothalamus are osmosensetive. *Nature* **287**: 154-156

McCabe, J.T., Kawata M., Sano Y., Pfaff S.W. and Desharnis R.A. 1990. Quantitative *in situ* hybridization to measure single-cell changes in vasopressin and oxytocin mRNA levels after osmotic stimulation. *Mol. Cell. Neurobiol.* **10**: 59-71

McCabe, J.T., Kao, T.-C. and Volkov, M.L. 1993. An assessment of the efficacy of *in situ* hybridization as a quantitative method by variance components estimation. *Microsc. Res. Tech.* **25**: 61-67

Meister, B. 1993. Gene expression and chemical diversity in hypothalamic neurosecretory neurons. *Mol. Neurobiol.* **7**: 87-110

Meister, B., Cortes, R., Villar, M.J., Schalling, M. and Hokfelt, T. 1990. Peptides and transmitter enzymes in hypothalamic magnocellular neurons after administration of hyperosmotic stimuli: comparison between messenger RNA and peptide/protein levels. *Cell Tissue Res.* **260**: 279-297

Mellström, B., Naranjo, J.R., Foulkes, N.S., LaFarga. M. and Sassone-Corsi, P. 1993. Transcriptional response to cAMP in brain: Specific distribution and induction of CREM antagonists. *Neuron* **10**: 655-665.

Merlie, J.P., Isenberg, K.E., Russell, S.D., and Sanes, J.R. 1984. Denervation supersensitivity in skeletal muscle: analysis with a cloned cDNA probe. *J. Cell Biol.* **99**: 332-335

Mohr. E. and Richter, D. 1990. Sequence analysis of the promoter region of the rat vasopressin gene. *Fedn Eur. Biochem. Socs. Lett.* **2**: 305-308

Montarolo, P.G., Goelet, P., Catellucci, V.F., Morgan, J., Kandel, E.R., and Schacher, S. 1986. A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in Aplysia. *Science* **234**: 1249-1254

Morgan, J.I., Cohen, D.R., Hempstead, J.L., Curran, T. 1987. Mapping patterns of c-fos expression in the central nervous system after seizure. *Science* **37**: 192-197

Morgan, J.I., and Curran, T. 1986. Role of ion flux in the control of c-fos expression. *Nature* **322**: 552-555

Morgan, J.I., and Curran, T. 1988. Calcium as a modulator of the immediate-early gene cascade in neurons. *Cell Calcium* **9**: 303-311

Morgan, J.I., and Curran, T. 1991. Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes *fos* and *jun*. *Annu. Rev. Neurosci.* **14**: 421-51

Morris, J.F., Nordmann, J.J. and Dyball, R.E.J. 1978. Structure-function correlation in mammalian neurosecretion. *Int. Rev. Exp. Pathol.* **18**: 1-95

Müller, R., Wagner, E.F. 1984. Differentiation of F9 teratocarcinoma stem cells after transfer of c-*fos* proto-oncogenes. *Nature* **311**: 438-442

Nakabeppu, Y., Ryder, K., and Nathans, D. 1988. DNA binding activities of three murine jun protein: Stimulation by Fos. *Cell* **55**: 907-15

Naranjo, J.R., Mellström, B., Achaaval, M. and Sassone-Corsi, P. 1991. Molecular Pathways of Pain: Fos/Jun-Mediate Activation of a noncanonical AP-1 site in the prodynorphin gene. *Neuron* **6**: 607-617

Nisenbam, L.K., Zigmond, M.J., Sved, A.F. and Avercrombie E.D. 1991. Prior exposure to chronic stress results in enhanced synthesis and release of hippocampal norepinephrine in response to novel-stressor. *J. Neurosci.* **11**:1478-1484

Nunez, D.J.R., Davenport, A.P., Emson, P.C. and Brown, M.J. 1989. A quantitative " in situ" hybridization method using computer-assisted image analysis. *Biochem. J.* **263**: 121-127

Offork, J., and Catterall, W.A. 1989. Electrical activity, cAMP, and cytosolic calcium regulate mRNA encoding sodium channel α subunits in rat muscle cells. *Neuron* **2**, 1447-1452

Palkovits, M. 1986. Afferents onto neuroendocrine cells. **Morphology of Hypothalamus and its Connections** (*Current Topics in Neuroendocrinology vol. ?*) D. Ganten and D. Pfaff, eds., Berlin: Springer-Verlag, pp. 197-222

Phillips, M.I. 1987. Brain angiotensin. In P.M. Gross (Ed.), *Circumventricular Organs and Body Fluids, Vol. I*, CRC Press, Florida, pp.163-182

Piechaczyk, M and Blanchard, J.-M. 1994. c-*fos* proto-oncogene regulation and function. *Crit. Rev. Oncol. Hematol.* **17**: 93-131.

Piette, J. and Yaniv, M. 1987. Two different factors bind to the a-domain of the polyoma virus enhancer, one of which also interacts with the SV-40 and c-*fos* enhancers. *EMBO. J.* 6: 1331-1337

Rauscher, F.J.III, Sambucetti, L.C., Curran, T., Distel, R.J., Spiegelman, B. 1988a. A common DNA binding site for fos protein complexes and transcription factor AP-1. *Cell* **52**: 471-480

Rauscher, F.J.III, Voulalas, P.J., Franza, B.R. Jr. and Curran, T. 1988b. Fos and Jun bind cooperatively to the AP-1 site: Reconstitution in vitro. *Genes Dev.* **2**: 1687-99

Rauscher, F.J.III, Cohen, D.R., Curran, T., Bos, T., Vogt, P. K., Bohmann, D., Tjian, R. and Franza, B.R., Jr. 1988c. Fos-associated protein p39 is the product of the *jun* proto-oncogene. *Science* **240**: 1010-1016

Reichlin, S., Ed. 1984. **The Neurohypophysis: Physiological and Clinical Aspects**. New York, Plenum Publishing Corp., p.1

Reid, I.A. and Schwartz , J. 1984. Role of vasopressin in the control blood pressure. In. L. Martini and W. F. Ganong, Ed.s., **Frontiers in Neuroendocrinology**, Vol. **8.** New York: Raven Press, pp. 177-197

Rettig, R.,Lang, R. E., Rascher, W., Unger, Th. and Ganten, D. 1982. Brain peptides and blood pressure regulation. *Clin. Sci.* **63**: 269s-283s

Reuss, L and Bello-Reuss, E (1983) Basic concepts of fluid, electrolyte, and renal physiology. IN: **The Kidney and Body Fluids in Health and Disease**, S. Klahr, Ed. New york: Plenum Medical Book Company, pp. 3-34.

Rhodes, C.H., Morrell, J.I., and Pfaff, D.W. 1981. Immunohisto-chemical analysis of magnocellular elements in rat hypothalamus: Distribution and numbers of cells containing neurophysin, oxytocin, and vasopressin. *J. Comp. Neurol.* **198**: 45-64

Roberts, M.M., Robinson, A.G., Fitzsimmons, M.D., Grant, F., Lee, W., Hoffman, G.E. 1993. c-fos expression in vasopressin and oxytocin neurons reveals functional heterogeneity with magnocellular neurons. *Neuroendocrinolgy* **57**: 388-400

Robertson, G.L. 1977. The regulation of vasopressin function in health and disease. *Recent Prog. Horm. Res.* **63**: 333-431

Rüther U., Wagner E.F., and Müller, R. 1985. Anlysis of the differentiation-promoting potential of inducible c-*fos* genes introduced into embryonal carcinoma cells. *EMBO J* **4**: 1775-1781

Ryder, K., Lau, L.F., and Nathans, D. 1988. A gene activated by growth factors is related to the oncogene v-jun. *Proc. Natl. Acad. Sci.* **85**: 1487-91

Ryder, K., Lanaha A., Perez-Albuerne, E. and Nathans, D. 1989. Jun-D: A third member of the Jun gene family. *Proc. Natl. Acad. Sci.* **86**: 1500-3

Sagar S.M., Sharp F.R. 1988a. Dehydration induces c-fos immunostaining in hypothalamic magnocellular neurons. *Neurosci. Abstracts* **14**: 307

Sagar S.M., Sharp F.R., Curran T. 1988b. Expression of c-fos protein in brain: Metabolic mapping at the cellular level. *Science* **244**: 1328-1331

Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysduel, J., Leutz, A., Beug, H., Vennstrom, B. 1986. The c-erb A protein is a high affinity receptor for thyroid hormone. *Nature* **324**: 635-637

Sassone-corsi. P., Sisson, J.C., and Verma I.M. 1988. Transcriptional autoregulation of the proto-oncogene *fos*. *Nature* **334**: 310-314

Schmale, H., Heinsohn, S., and Richter, D. 1983. Structural organization of the rat gene for the arginine vasopressin-neurophysin precursor. *EMBO J.***2**: 763-767

Schönthal, A., Büscher, M., Angel. P. et al. 1989. The Fos and Jun/AP-1 proteins are involved in the down regulation of *fos* transcription. *Oncogene* **4**: 629-636

Schrier, R.W. (ed.) 1985. **Vasopressin**. New York: Raven Press, pp. 1-577.

Schütte, J., Viallet, J., Nau, M., Segal, J., Fedorko, J., and Mina, J. 1989. *jun*-B inhibits, and c-fos stimulates the transforming and trans-activating activities of c-*jun*. *Cell* **59**: 987-997

Setoyama, C., Frunzio, R., Liau, G., Mudryi, M., DeCrombrugghe, B. 1986. Transcriptional activation encoded by the v-fos gene. *Proc. Natl. Acad. Sci.* **83**: 3213-3217

Sharp, F.R., Sagar, S. M., Hicks, K., Lowenstein, D., and Hisanaga, K. 1991: c-fos mRNA, Fos, and Fos-related antigen induction by hypertonic saline and stress. *J. Neurosci.* **11**: 2321-2331

Sheng, M., Greenberg, M.E. 1990. The Regulation and function of *c-fos* and other immediate early genes in the nervous system. *Neuron* **4**: 477-485

Sherman T.G., Civelli O., Douglass J., Jerbert E. and Watson S.J. 1986. Coordinate expression of the hypothalamic pro-dynorphin and pro-vasopressin mRNAs with osmotic stimulation. *Neuroendocrinology.* **44**: 222-228

Sherman T.G., Day R., Civelli O., Douglass J., Herbert E., Akil H, and Watson S.J. 1988. Regulation of hypothalamic magnocellular neuropeptides and their mRNAs in the Brattleboro rat: coordinate responses to further osmotic challenge. *J. Neurosci.* **8**: 3785-3796

Silverman, A.J. and Zimmerman, E.A. 1983. Magnocellular neurosecretory system. *Annu. Rev. Neurosci.* **6**: 357-380

Sladek, C.D., and Armstrong, W.E. 1985. Neural pathways subserving osmotic control of vasopressin release. In R.W. Schrier (Ed.) *Vasopressin*, Raven, New York, pp.333-342

Sonnenberg, J.L., Mitchelmore, C., Macgregor-Leon, P.F., Hempstead, J., Morgan, J.I. and Curran, T. 1989. Glutamate receptor agonists increase the expression of Fos, Fra, and AP-1 DNA binding activity in the mammalian brain. *J. Neurosci. methods* **24**: 72-80

Stehelin, D., Varmus, H.E., Bishop, J.M., and Vogt, P.K. 1976. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. *Nature* **260**: 170-173

Subramaniam, M., Schmidt, L.J., Crutchfield III, C.E., and Getz, M.J. 1989. Negative regulation of serum responsive enhancer elements. *Nature* **340**: 64-66

Swaab, D.J., Nijveldt, F. and Pool, C.W. 1975. Distribution of oxytocin and vasopressin in the rat supraoptic and paraventricular nucleus. *J. Endocrinol.* **67**: 461-462

Swanson, L.W. 1986. Organization of the mammalian neuroendocrine system. In: **Handbook of Physiology, Section 1: The Nervous System, Volume IV. Intrinsic Regulatory Systems of the Brain.** American Physiological Society, Bethesda, MD, Chapter 6, pp. 317-363

Swanson, L.W. and Sawchenko, P.E. 1983. Hypothalamic integration: Organization of the paraventricular and supraoptic nuclei. *Annu. Rev. Neurosci.* **6**: 269-324

Treisman, R. 1985. Transient accumulation of c-*fos* RNA following serum stimulation requires a conserved 5' element and c-*fos* 3' sequence. *Cell* **42**: 889-902

Van Beveren, C., Verma, I.M. 1985. Homology among oncogenes. *Curr. Top. Microbiol. Immunol.* **123**: 73-98

Van Tol J.J.M., Vorhuis D.Th.A.M. and Burbach J.P.H. 1987. Oxytocin gene expression in discrete hypothalamic magnocellular cell groups is stimulated by prolonged salt loading. *Endocrinology* **120**: 71-76

Verma, I.M., Sassone-Corsi, P. 1987. Proto-oncogene fos: Complex but versatile regulation. *Cell* **51**: 513-516

Vogt, P.K. and Bos, T.J. 1990. *jun*: Oocogene and transcription factor. *Adv. Cancer Res.* **55**: 1-32

Wakerly, J.B. 1987. Electrophysiology of the central vasopressin system. In: *Vasopressin: Principles and Properties*, D.M. Gash and G.J. Boer, eds., Plenum Press, NY, pp.211-256.

Weinberger, C., Thompson, C.C., Ong, E.S., Lebo, R., Groul, D.J., Evans, R.M. 1986. The c-erb A gene encodes a thyroid hormone receptor. *Nature* **324**: 641-643

Weindl, A. 1985. Neuroanatomical pathways related to vasopressin. In: **Neurobiology of Vasopressin** (*Current Topics in Neuroendocrinology vol. 4*) D. Ganten and D. Pfaff (eds.). Springer-Verlag, Berlin, pp. 127-155

Zerial, M., Toschi, L., Ryseck, R.P., Schuermann, M., Muller, R. and Bravo, R. 1989. The product of a novel growth factor activated gene, fosB, interacts with Jun proteins enhancing their DNA binding activity. *EMBO J.* **8**: 805-13